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Award Number:

W81XWH-09-2-0081

TITLE:

EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE

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REPORT DATE:

September 2012

TYPE OF REPORT:

Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-09-2012	Annual	09/01/2011 – 08/31/2012

4. TITLE AND SUBTITLE

EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-2-0081

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

SRI
Menlo Park, CA 94025-3493

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During Year 3, results continued to accumulate that are consistent with the hypothesis that disfacilitation of wake-promoting systems by the hypocretin (Hcrt) receptor antagonist almorexant (ALM) results in less functional impairment than the inhibition of neural activity produced by the benzodiazepine receptor agonist zolpidem (ZOL). Measures of both spatial reference memory (Task 2a) and spatial working memory (Task 2b) in rodents treated with ALM were mostly indistinguishable from vehicle whereas impairments were clearly evident under ZOL. Similarly, wake-active Hcrt neurons can be recruited in the presence of ALM after sleep deprivation but not in the presence of ZOL (Task 3a). Conversely, although both drugs activate sleep-active cortical neurons, sleep-active cells are more strongly activated by ZOL (Task 4a). Lesions of the basal forebrain (BF), a wakefulness-promoting area, potentiated the hypnotic effect of ZOL without affecting the response to ALM (Task 3b), indicating different neural pathways underlie the actions of these two drugs. ALM promoted adenosine and glutamate release in the BF (Task 4b) whereas ZOL promoted GABA release, particularly during waking. An *In Vivo* Cellular Neurophysiology Laboratory was established to perform the electrophysiology and optogenetic experiments (Tasks 6a-c).

15. SUBJECT TERMS
Sleep, performance, drug, neurotransmitter, hypocretin, orexin, benzodiazepine, zolpidem, neurochemistry, microdialysis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Thomas S. Kilduff, Ph.D.
U	U	U	UU	44	19b. TELEPHONE NUMBER *(include area code)* 650-859-5509

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"Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance"
USAMRAA Grant W81XWH-09-2-0080
DR080789P1
Year 3: 8/1/11 to 7/31/12
Thomas S. Kilduff, Ph.D., Principal Investigator

INTRODUCTION

Almorexant (ALM) is a hypocretin/orexin (Hcrt) receptor antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The overall hypothesis that underlies this research is that ALM produces less functional impairment than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. Whereas the human study component will establish whether ALM is superior to ZOL in neurocognitive tests, the animal studies will compare the neural circuitry that underlies the activity of these compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

BODY

Task 2. *Test the hypothesis that rodents receiving ZOL will show greater neurocognitive impairment than those receiving ALM or PBO.*
2a. Assessment of Almorexant effects on spatial reference memory in rats.
2b. Assessment of Almorexant effects on spatial working memory in rats.
2c. Assessment of Almorexant effects on psychomotor vigilance in rats.
2d. Synthesis of Almorexant.

Progress – Task 2a: All data collection for Task 2a is complete and most data analysis is complete. All water maze (WM) data for the effects of ALM on spatial reference memory are reported here. We have assessed the effects of ALM on spatial reference memory both following undisturbed and sleep deprivation conditions.

Methods: All rats were given a minimum of 3 wks for recovery from surgery and each rat was recorded for a 24 h period to determine basal sleep/wake patterns. Assessment the effects of ALM on spatial reference memory occurred on 2 consecutive days. On day 1, the acquisition of the task occurred in one session consisting of 8-12 consecutive WM trials with a 60 second (s) inter-trial interval. On the following day, rats were either left undisturbed and dosed with ALM, ZOL or vehicle (VEH) 6 h into their active period (ZT18) or kept awake for the first 6 h of the dark and then dosed. Subsequent to dosing, rats were left undisturbed for 90 min and then a retention probe trial was performed. For this test, the platform was removed from the WM and the rats were allowed to swim and search for the platform for 30 s. Parameters measured during the retention probe trial included the time and distance traveled in the quadrant of the WM where the platform had been on the acquisition day, as well as the latency and the number of entries into the target quadrant. EEG and EMG recordings were analyzed from the beginning of lights

out (ZT12) until initiation of the WM test (7.5 h later). For more details on our experimental procedures, please see the full protocols in our original proposal.

Results: As reported previously, both ALM (100 mg/kg i.p and p.o.) and ZOL (30 mg/kg i.p. and 100 mg/kg p.o.) had significant sleep-promoting effects. Waking (W) was decreased and non-rapid eye movement sleep (NR) increased by ALM and ZOL compared to vehicle. Although NR was increased to a greater extent following ZOL than ALM, rapid eye movement (REM) sleep was increased significantly more by ALM than by ZOL. Importantly, for the 60 min prior to WM testing, the ALM and ZOL groups of rats slept equivalent amounts; the differences between the sleep-promoting effects of ALM and ZOL occurred primarily during the first 30 min following drug administration. Confirming our previous findings, ZOL significantly reduced the latency to sleep onset compared to vehicle and ALM.

During the WM probe trial following undisturbed conditions, rats administered ZOL showed impairments in all parameters measured compared to rats administered VEH or ALM whereas ALM was indistinguishable from VEH for all measures (Figure 1). Following ZOL, rats swam significantly less (Fig. 1A), took longer to reach the target zone (Fig. 1C), spent less time in the target zone (Fig. 1E), and entered the target zone less frequently (Fig. 1G) compared to rats administered VEH or ALM.

During the WM probe trial following the 6 h sleep deprivation, ALM was again indistinguishable from VEH for all measures. Following ZOL, rats swam significantly less (Fig. 1B) and took longer to reach the target zone (Fig. 1D) compared to rats administered VEH or ALM. However, time spent in the target zone (Fig. 1F) and the frequency of entering the target zone (Fig. 1H) was not different from rats administered VEH or ALM.

These results are consistent with the hypothesis that, although both ALM and ZOL are effective hypnotic medications, rats would show less functional impairment following ALM than following ZOL treatment.

Progress – Task 2b: Similar to the assessment of the effects of ALM on spatial reference memory in Task 2a, we have proposed to assess the effects of ALM on spatial working memory following both undisturbed and sleep deprived conditions in Task 2b. All data collection is complete for the undisturbed condition and the data is reported here. For the sleep-deprived condition, all rats have been implanted and the tests are currently being completed.

Methods: The general protocol for assessing spatial working memory is as follows. Approximately one week before spatial working memory testing, rats are trained in a standard WM protocol as described in Task 2a. During this training, the location of the hidden platform remains constant across all trials. A baseline sleep recording occurs following training but prior to testing, in order to assess the undisturbed sleep-wake patterns of each rat. On the experimental day, rats are either left undisturbed prior to administration of drug at ZT18 or sleep deprived for 6h prior to drug administration. Rats are then left undisturbed until testing 60 min later.

The spatial working memory task consists of 6 pairs of trials. In the first trial, a cued platform marked with a flag is placed in one of 6 positions in the tank. Rats are released facing the wall from one of the 3 quadrants that does not contain the platform and are allowed 120 s to locate the cued platform before the researcher guides the rat to the platform. After 15 s on the

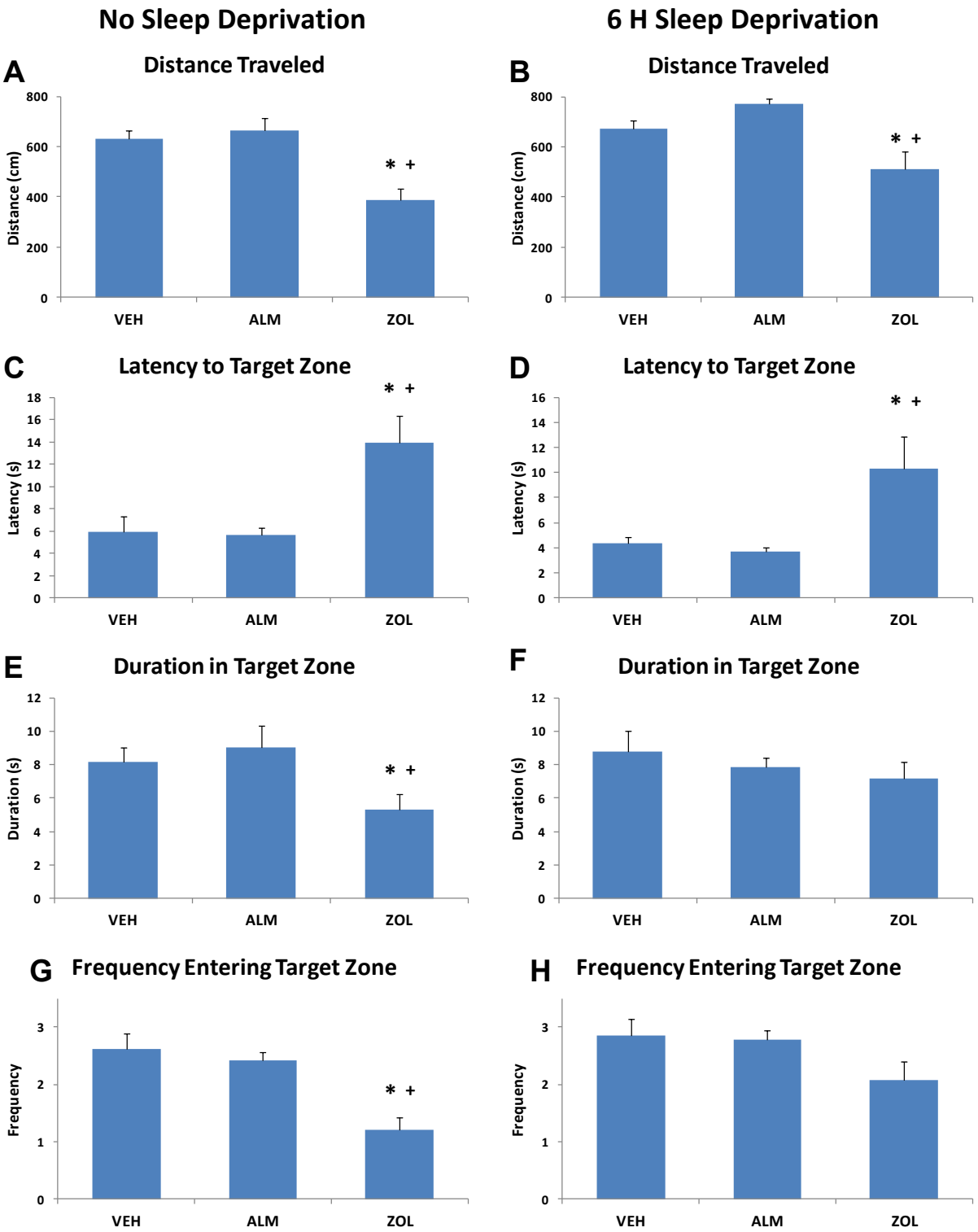

Figure 1. Measures of spatial reference memory during the WM probe trial. For the non-sleep deprived condition (left column), rats were left undisturbed for the first 6 h after lights off. Rats were then dosed and tested 90 min later. For the sleep deprived condition (right column), rats were kept awake for the first 6 h after lights off and then dosed. Following dosing, rats were left undisturbed and tested 90 min later. **A, B.** Distance traveled during the 30 s probe trial. Rats administered ZOL swam significantly less than rats administered vehicle or ALM following both undisturbed and sleep deprived conditions. **C, D.** Latency to

entry into the target zone. Rats administered ZOL took significantly longer time to enter the target zone compared to rats administered vehicle or ALM following both undisturbed and sleep deprived conditions. **E, F.** Time spent in the target zone. Rats administered ZOL spent significantly less time in the target zone compared to rats administered vehicle or ALM only following undisturbed conditions. **G, H.** Frequency of entry into the target zone. Rats administered ZOL entered the target zone significantly fewer times compared to rats administered vehicle or ALM only following undisturbed conditions.
* = significantly different from vehicle; **+** = significantly different from ALM.

platform, rats are removed from the WM and placed in a holding cage. The flag is then removed but the platform remains in the same location as in the first trial. Following a delay of 1, 5, or 10 min in the holding cage, the rat is placed back in the tank in one of the two quadrants that did not contain the platform and was not the starting quadrant from the first trial. Once the rats finds the platform, it is removed after approximately 5 s on the platform and placed back in a holding cage for 10 min before a new pair of trials with a novel platform location is given. The order of delays is counterbalanced so that each rat is tested twice at 1, 5, or 10 min delays between the cued and hidden platforms. Test measures are velocity, time and distance traveled to locate the platform during all tests.

Results: During the WM test trials following undisturbed conditions, rats administered ZOL showed impairments in almost all parameters measured compared to rats administered VEH or ALM whereas ALM was indistinguishable from VEH for all measures (Figure 2). Following ZOL, rats swam less (Fig. 2A), took longer to find the platform (Fig. 2B), and swam more slowly (Fig. 2C) compared to rats administered VEH or ALM. Importantly, following ZOL, rats failed to find the platform significantly more often than following ALM or VEH (Figure 3).

To investigate potential mechanisms underlying whether the platform was found or not, we analyzed distance traveled and velocity when the platform was found and when it was not found (Figure 4). During the few trials where the platform was not found following ALM or VEH, rats swam greater distances than when they found the platform (Fig. 4A vs. Fig. 4B), indicative of the searching behavior observed under these conditions. Following ZOL, however, distance traveled was not greatly different between the trials where the platform was located and when it was not. This measure indicates a relative lack of searching behavior in rats following ZOL treatment. In contrast, velocity did not differ for the trials when the platform was found compared to when it was not found for any drug condition. These results support our initial hypothesis that rats would perform more poorly (i.e., have greater functional impairment) following ZOL than following ALM. These results demonstrate that ALM impairs the performance of rats less than ZOL does in this spatial reference memory task.

Progress – Task 2c: The equipment for the rodent psychomotor vigilance (rPVT) test has been ordered the experiments are to begin immediately following the completion of the WM experiments. We expect to order rats and begin training by early September.

Progress – Task 2d: Synthesis of ALM. COMPLETED

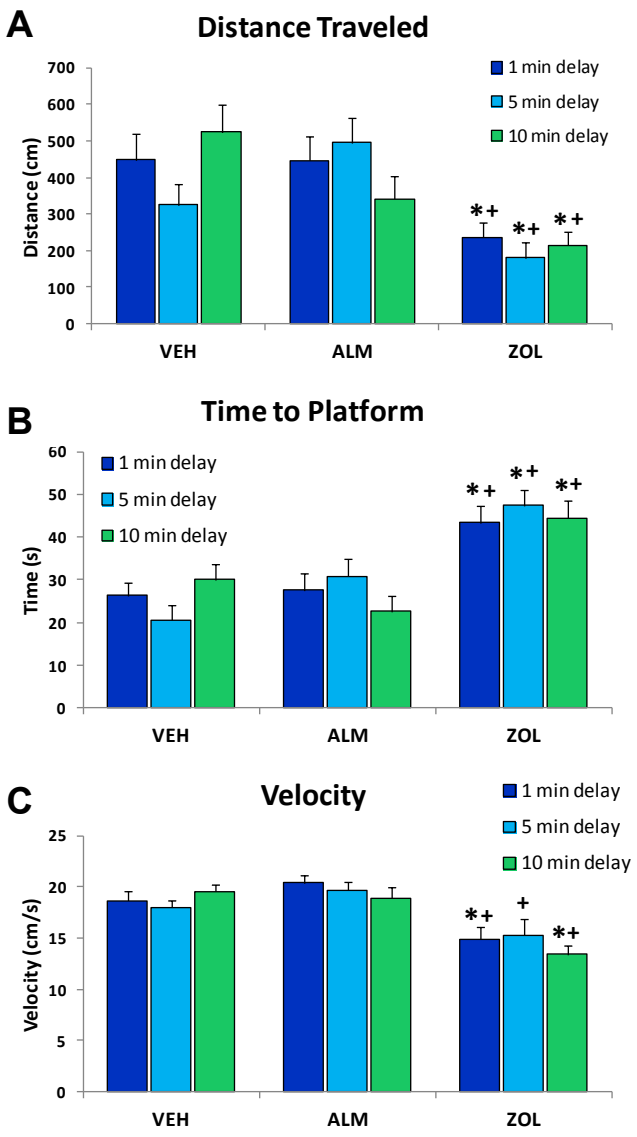

Figure 2. Measures of spatial working memory during the WM task. **A.** Distance traveled during the 60 s test trial. Rats administered ZOL swam significantly less than rats administered vehicle or ALM. **B.** Time taken to find platform during the 60 s test trial. Rats administered ZOL took significantly longer to find the platform than rats administered vehicle or ALM. **C.** Velocity of the rat during the 60 s test trial. Rats administered ZOL swam significantly slower than rats administered vehicle or ALM.
* = significantly different from vehicle;
+ = significantly different from ALM.

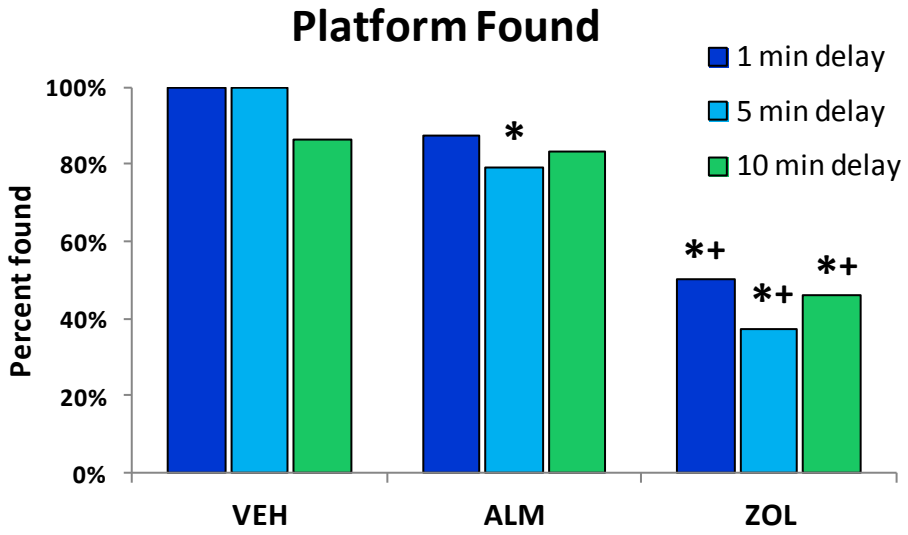

Figure 3. Percentage of trials during which rats found the platform during the WM test trial measuring spatial working memory. Rats administered ZOL found the platform significantly less than rats administered vehicle or ALM. * = significantly different from vehicle; **+** = significantly different from ALM.

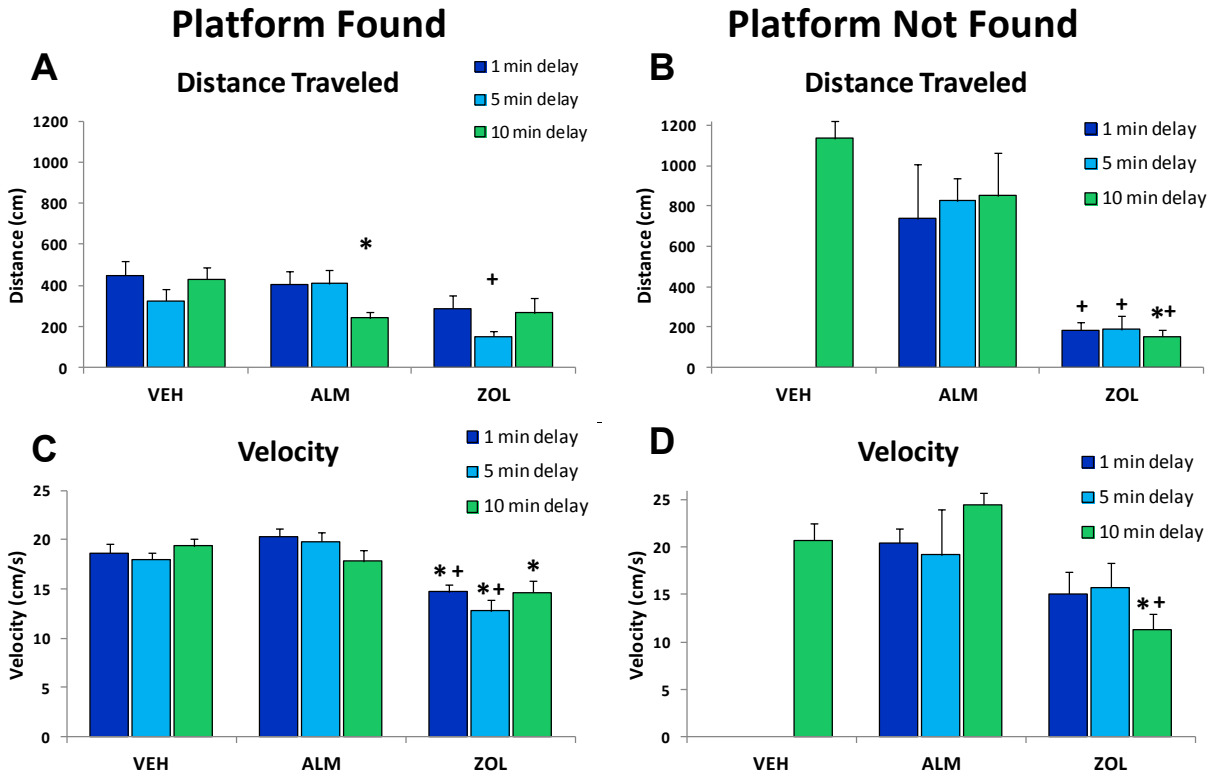

Figure 4. Distance traveled and velocity of the rats during the spatial working memory WM task separated by whether the platform was found (left panels) or not found (right panels) during the trial. **A, B.** Distance traveled during the 60 s test trial. Rats administered VEH or ALM swam further during the trials when the platform was not found than when it was found. Rats administered ZOL swam similar distances whether the platform was found or not. **C, D.** Velocity during the 60 s test trial. The velocity of the rats was similar whether the platform was found or not during all three drug conditions. * = significantly different from vehicle; **+** = significantly different from ALM.

Task 3. *Test the hypothesis that the Hcrt antagonist ALM induces sleep by selectively disfacilitating the activity of the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems whereas the BzRA ZOL causes a generalized inhibition of the brain.*
3a. Double-label immunohistochemistry with Fos and phenotypic markers.
3b. Assessment of hypnotic efficacy in saporin-lesioned rats.
3c. Assessment of hypnotic efficacy in transgenic mice.

Progress - Task 3a: As described in the last Progress Report, we generated a cohort of animals dosed intraperitoneally with 100 mg/kg ALM, 30 mg/kg ZOL, or VEH for histological studies. This year we processed 46 additional rats dosed with 1 ml of 100 mg/kg ALM p.o., 1 ml of 100 mg/kg ZOL p.o. or 1 ml of VEH. This dosing regimen was adapted to match the dosage and delivery used for the behavioral studies in Task 2.

To assess the influence of ALM and ZOL on the activity of sleep/wake regulatory neurons, we performed an immunohistochemical study using c-Fos as a marker of neuronal activity. Rats were administered ALM (100 mg/kg p.o., n=16), ZOL (100 mg/kg p.o., n=16), or vehicle (n=14) at ZT18. Half of the animals in each drug treatment condition were allowed to sleep for 1.5h after dosing, whereas the remaining rats were sleep deprived by gentle handling (SD). All animals were then deeply anesthetized, perfused and the brains sectioned. Double-label immunohistochemistry for Fos, a marker of functional activity, and the neuropeptide hypocretin (Hcrt; a "wake-active" hypothalamic neuronal population) was performed in coronal brain sections at the level of the lateral hypothalamus. The results confirmed the main findings reported in the last Progress Report: wake-active Hcrt neurons showed higher levels of Fos expression after SD than after the undisturbed condition only in the ALM-treated rats (Fig. 5) whereas there was no such difference for the ZOL-treated rats. These results indicate that activation of the Hcrt neurons by SD is unimpaired in the presence of ALM whereas ZOL appears to block such activation.

To assess Fos expression in other populations of wake-active neurons, we developed staining protocols for immunohistochemical staining of adenosine deaminase, serotonin, dopamine beta-hydroxylase, and choline acetyltransferase. During Year 4, double immunochemistry for Fos and these markers will be carried out using the tissue already processed as described above.

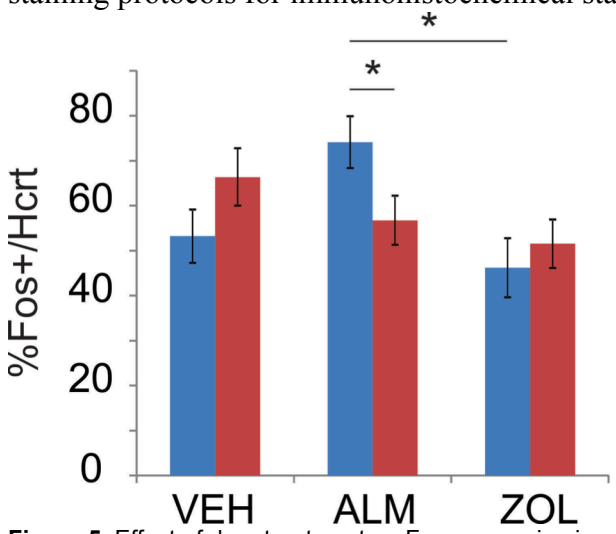

Figure 5. Effect of drug treatment on Fos expression in wake-active Hcrt neurons. Blue bars depict group means for sleep deprived rats, red bars for undisturbed rats. * = p< 0.05 for pairwise comparisons following ANOVA.

Progress - Task 3b: To test the hypothesis that ALM induces sleep by selectively disfacilitating the activity of cholinergic wake-promoting systems, 16 male Sprague-Dawley rats were instrumented for telemetric recording of EEG, EMG, locomotor activity (LMA) and core body temperature (CBT), and were injected bilaterally in the basal forebrain with neurotoxin 192-IgG-saporin (BFx; n=8) or sterile saline (Sham; n=8). Injections were delivered stereotaxically via a calibrated glass micropipette using a pressurized air delivery system (Picospritzer, Parker-Hannefin). Rats were given 3 wk recovery to ensure that neuronal degeneration was complete. One BFx rat was removed from the study due to postoperative weight loss (final n=7). Prior to dosing, rats were recorded for a 24 h undisturbed baseline, sleep-deprived for 6 h starting at lights-off, and then allowed to recover undisturbed. After an 18 h recovery period, rats were given p.o. ALM 30, 100 and 300 mg/kg, ZOL 10, 30 and 100 mg/kg, and VEH at lights-off (ZT 12) in balanced order with at least 3 d between treatments. EEG, EMG, LMA and CBT were recorded continuously throughout experimentation.

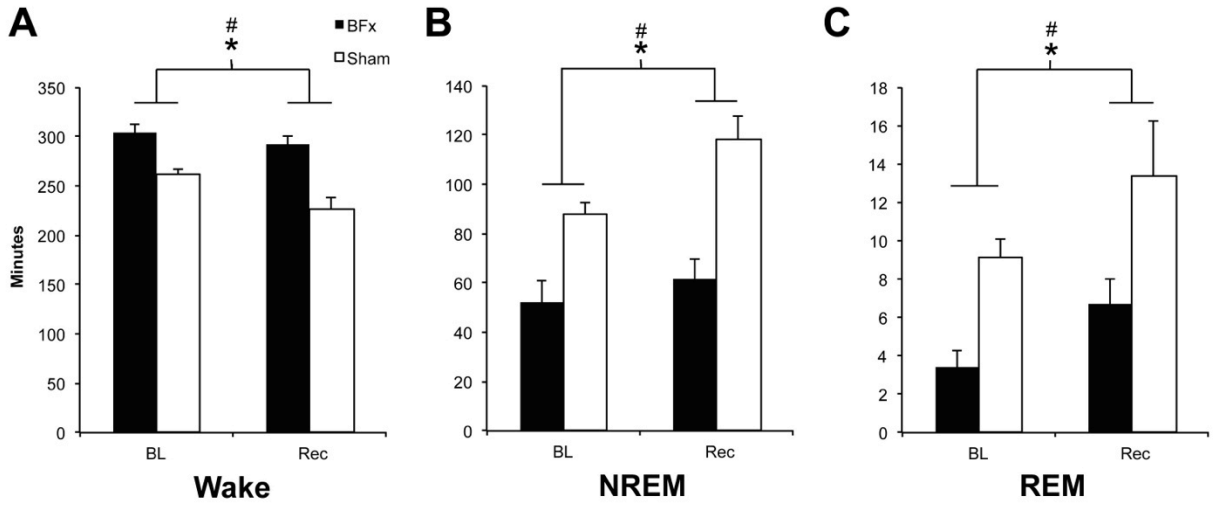

Figure 6. Cumulative Wake, NREM and REM sleep time in minutes for BF-lesioned (black) and Sham-operated (white) rats at baseline and after 6 h sleep deprivation (SD) in the dark phase. Bars represent total time spent in state from ZT 18 - ZT 23. *, significant main effect of lesion; #, significant main effect of SD. All effects considered significant at $p < 0.05$.

BFx decreased NREM and REM sleep and increased waking in the dark phase compared to Sham at baseline and following 6 h SD (Fig 6). By contrast, baseline sleep in the light phase was unaffected (not shown). While this stimulatory effect is at odds with the BF's proposed role as an arousal-promoting system, the BF is also known to mediate homeostatic increases in sleep, such as following prolonged wakefulness. Thus, the lesions, by destroying an important site of integration for homeostatic sleep cues, likely decreased the ability to respond to elevated sleep pressure during their normal active phase.

To control for reduced baseline sleep in BFx animals compared to controls, sleep from the drug dosing regimen was normalized to each individual's vehicle condition. ALM and ZOL increased NREM sleep over VEH as expected, but did so differently in BFx and Sham rats (significant drug x lesion x ZT interaction, $F_{30,390} = 2.94$, $p < 0.0001$; Fig. 7B-D). ALM increased NREM sleep to a similar extent in BFx and Sham rats (150% - 200% of vehicle), whereas ZOL induced substantially larger increases in NREM sleep in BFx (300% - 450%) than in Sham rats (150% - 200%; Fig. 7E-G). BFx rats had fewer NREM bouts than Shams when given vehicle and ALM, but similar bout numbers when given ZOL. NREM bout length was unaffected by

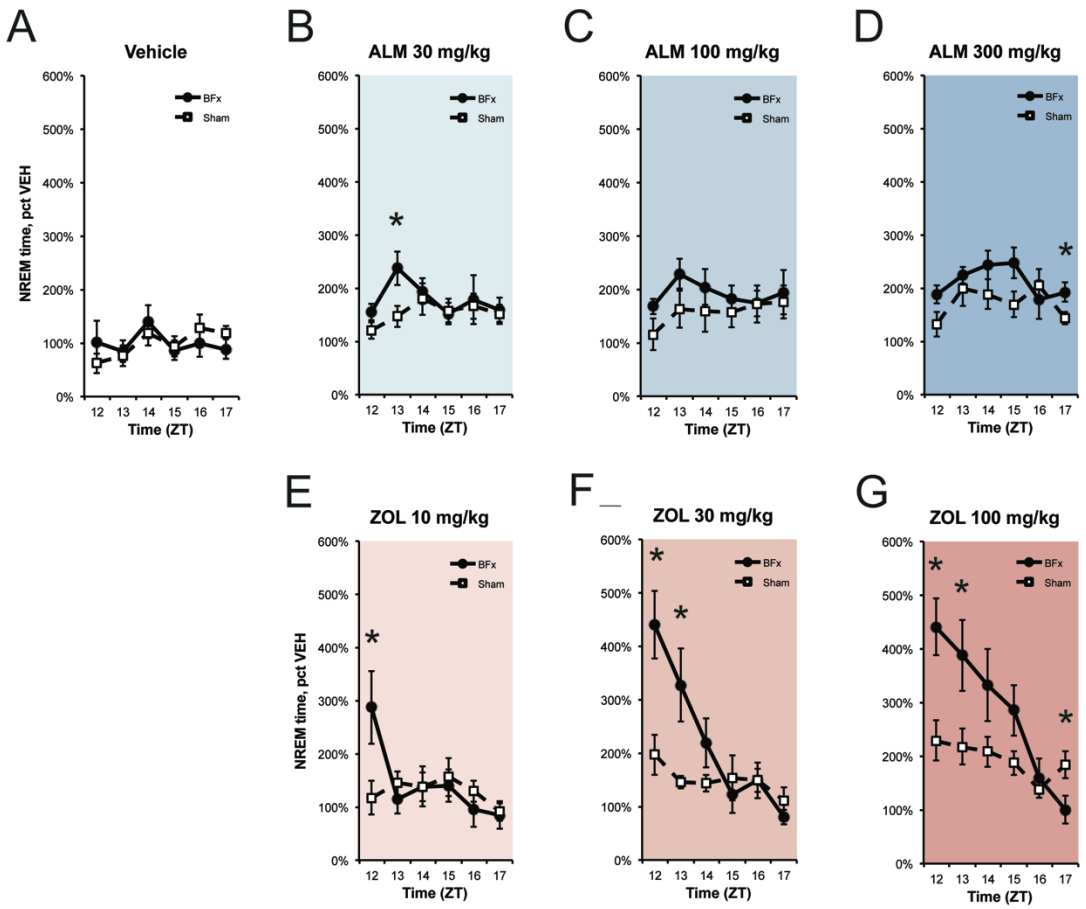

Figure 7. Hourly NREM sleep time in BFx (black) and Sham-operated (white) rats for 6 h following administration of VEH(A), ALM (B-D, blue bkgd) and ZOL (E-G, red bkgd) at lights-out (ZT12). For each individual, NREM time per h was normalized to the 6h-mean NREM time for VEH. Asterisks indicate significant effect of lesion; all effects considered significant at $p<0.05$.

ALM, but was increased by ZOL; interestingly, this increase was smaller in BFx rats than in Shams. REM sleep was increased by ALM but not ZOL, and BF lesions did not alter the response to either drug. These data suggest that decreased cholinergic tone in BFx rats may make them hypersensitive to ZOL without affecting the response to ALM.

In pilot studies, we verified our microinjection technique and the dosage for lesioning the wake-promoting noradrenergic locus coeruleus (LC) using anti-DBH-saporin (DBH-SAP). DBH-SAP was infused into the 3rd ventricle using a Hamilton syringe with a 35-gauge stainless steel needle coupled to a digital microinjection pump (World Precision Instruments, Sarasota FL). Infusions of 2.5 µg – 5 µg DBH-SAP effectively lesioned the LC (Fig. 8B), whereas infusions of 3 µg 192-IgG-SAP (Fig. 8A) or sterile water (not shown) left the LC intact. EEG implant and lesion surgery is underway, with behavioral assessment and drug response to follow. Hcrt-SAP, which we proposed to use to lesion the histaminergic TMN, is no longer commercially available. We are investigating possible alternate sources of this compound.

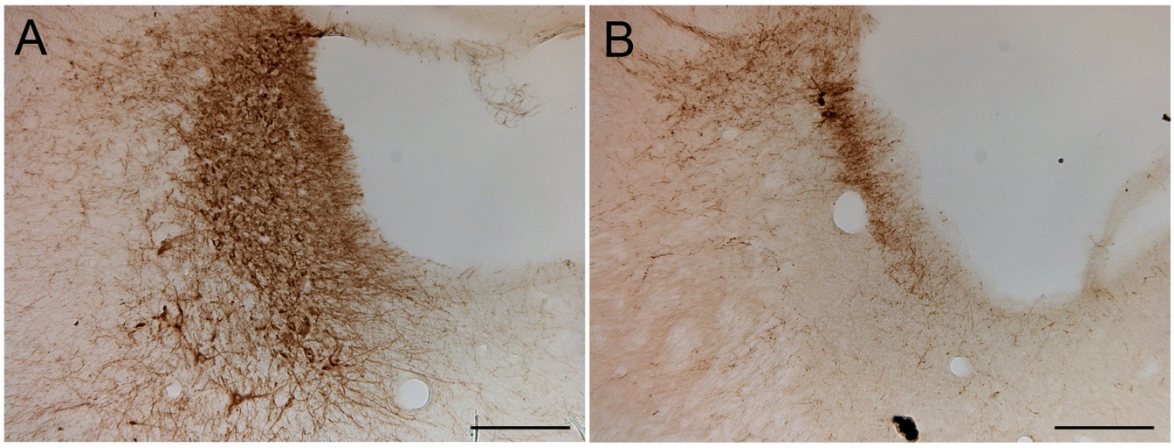

Figure 8. DBH-immunoreactivity in the locus coeruleus (LC) of rats injected with the selective neurotoxins 192-IgG saporin, which targets cholinergic neurons in the basal forebrain (A), or DBH-saporin, which targets noradrenergic neurons (B). Scale bar = 200 µm.

Progress - Task 3c: We identified an alternate source for *Pet1-Lmx1b(ffp)* mice; we are now awaiting importation of these mice from the University of Iowa, and *Dbh* KO mice from the University of Pennsylvania.

Task 4. *Test the hypothesis that ALM, but not ZOL, induces sleep by facilitating the mechanisms that underlie the transition to normal sleep.*
4a. Effects of ALM and ZOL on sleep-active brain areas.
4b. BF adenosine (ADO) release in response to oral ALM and ZOL.
4c. BF adenosine (ADO) release in response to ALM and ZOL by dialysis.

Progress - Task 4a: To match the doses and drug delivery route used in the Aim 2 experiments, we processed 46 more rats for histological studies as described for Task 3a. Double immunohistochemistry for the marker of functional activity, Fos, and the enzyme neuronal nitric oxide synthase (nNOS), a marker for sleep-active cortical neurons, was performed in coronal sections at the level of the anterior commissure. The results confirmed the data obtained from different drug dosing regimens reported in the last Progress Report. SD inhibited Fos expression in these sleep-active neurons in all drug conditions (Fig. 9). If the rats were allowed to sleep, a significantly higher proportion of cortical nNOS neurons expressed Fos for the groups dosed with either ALM or ZOL. This result indicates that neither ALM or ZOL directly activate cortical nNOS neurons, rather, activation of cortical nNOS neurons is permitted by sleep, which is promoted by both compounds. We also found that a smaller proportion of cortical nNOS neurons expressed Fos in animals dosed with ALM than in animals dosed with ZOL. This might be

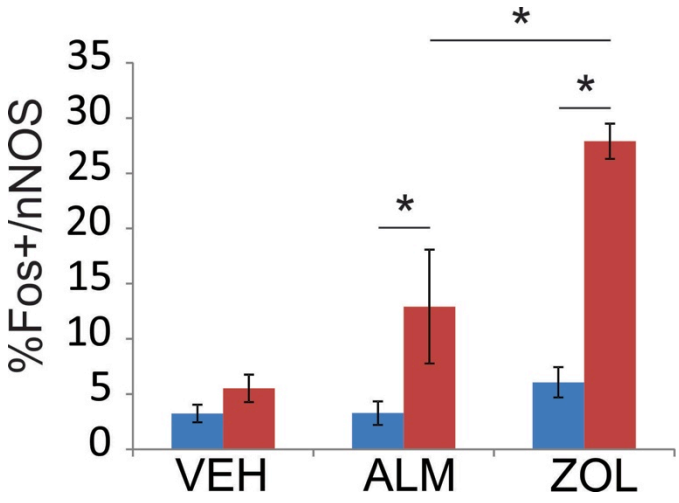

Figure 9. Effect of drug treatment on Fos-expression in sleep-active cortical nNOS neurons. Blue bars depict group means for sleep deprived rats, red bars for undisturbed rats. * = p< 0.05 for pairwise comparisons following ANOVA.

due to the delayed sleep onset induced by ALM relative to ZOL, resulting in a smaller amount of cumulative sleep time in the 1.5 h before perfusion.

To test whether there is a dose-response effect of ZOL-induced activation of cortical nNOS neurons, we added a group of 8 rats that were dosed with 30 mg/kg ZOL p.o. and again either sleep-deprived or left undisturbed for 1.5h until perfusion. Figure 10 compares the effects of this dose with the 100 mg/kg ZOL p.o. and VEH as described above. In contrast to the higher dose, 30 mg/kg ZOL p.o. did not increase the activation of sleep-active cortical nNOS neurons significantly, indicating a dose-response relationship between ZOL and activation of this neuronal population.

To test whether sleep pressure would influence the efficacy of ZOL to induce Fos in cortical nNOS neurons, we performed an additional experiment in 21 rats. The rats were dosed with either 100 mg/kg p.o. ZOL or VEH at ZT 12, when sleep pressure naturally is low. Half of the rats were sleep deprived for 6 h before dosing to increase sleep pressure. Two hours after dosing, all rats were perfused, brains were processed, and immunohistological double-labeling for Fos and nNOS was performed in coronal sections at the level of the anterior commissure. For both vehicle and ZOL-treated rats, the proportion of Fos-expressing cortical nNOS neurons was significantly higher when the rats had increased sleep pressure (Fig. 11). This result is consistent with our results presented in Figure 9 that ZOL is permissive for activation of cortical nNOS neurons but does not activate them directly, rather, sleep pressure is needed for direct activation.

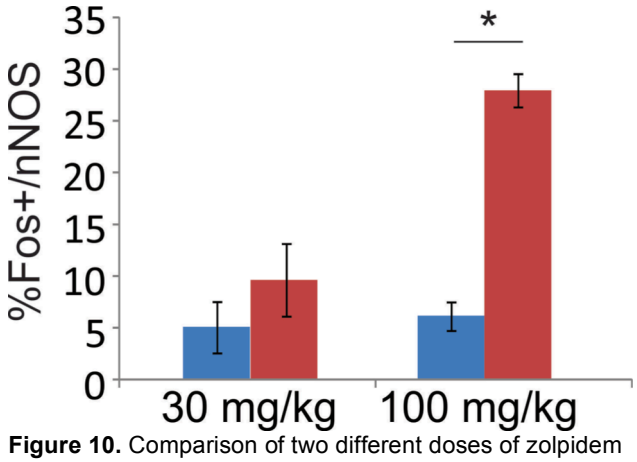

Figure 10. Comparison of two different doses of zolpidem on Fos-expression in sleep-active cortical nNOS neurons. Blue bars depict group means for sleep deprived rats, red bars for undisturbed rats. * = p< 0.05 for pairwise comparisons following ANOVA.

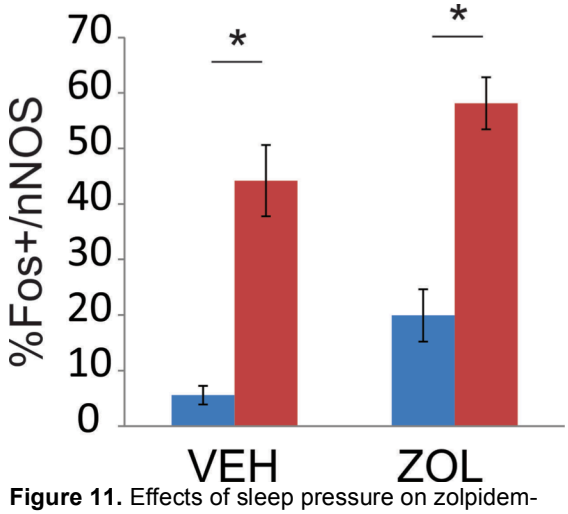

Figure 11. Effects of sleep pressure on zolpidem-induced Fos expression in sleep-active cortical nNOS neurons. Blue bars depict group means for animals with low sleep pressure, red bars for high sleep pressure. * = p< 0.05 for pairwise comparisons following ANOVA.

Progress – Task 4b: Task 4B was undertaken to study the effects of oral ALM and ZOL on basal forebrain (BF) adenosine (ADO) release, glutamate (GLU), and GABA release during sleep and wakefulness. We tested the hypothesis that oral ALM induces sleep by facilitating the mechanisms that underlie the transition to normal sleep. In contrast to ZOL, which affects $GABA_A$ receptors that are widely distributed in the CNS, we hypothesize that ALM acts through blockade of post-synaptic Hcrt receptors, thereby disfacilitating excitation in the BF. We used *in vivo* microdialysis and HPLC analyses to examine BF glutamate, GABA, and ADO efflux following oral ZOL (10 mg/kg), ALM (100 mg/kg), or placebo (VEH) combined with behavioral sleep analyses.

Experimental design. Male Sprague-Dawley rats were implanted with chronic recording devices (F40-EET, Data Sciences Inc., St Paul, MN) for continuous recordings of EEG, EMG, CBT, and LMA via telemetry as described previously (Morairty et al., 2008). Rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) for surgical implantation of a unilateral, stainless steel 26-gauge guide cannula aimed at the BF for microdialysis recovery of ADO, glutamate, and GABA. BF coordinates relative to bregma were P -0.3, L +2.0, V –5.0 (Paxinos and Watson, 2009).

Animals were given a 2-3 wk post-surgical recovery period prior to entering the experimental paradigm, conditioned to the microdialysis chamber, and handled for at least 30 min every day for 1 wk prior to the onset of the experiment to limit stress on the day of the experiment from exposure to a novel environment. A microdialysis probe was inserted through the guide cannula 16 h prior to the onset of the experiment day and continuously perfused with aCSF. At the start of the experiment (4.5 h into the dark period, ZT16.5), three 30 min baseline samples (1 µL/min flow rate, 30 µL TV) were collected from freely-moving animals to assess basal levels of ADO, GLU, and GABA while baseline EEG, EMG, T_b and LMA were collected to assess behavior. Each rat received one treatment in random order (washout period minimum 1 week) with parallel microdialysis sampling of the BF. Drug doses included ALM (100 mg/kg), ZOL (10 mg/kg) and VEH. One of three drugs was subsequently given p.o. to the animals 6h into the dark period (the rats' normal active period) (ZT18), and six additional 30 min samples were collected to assess the effects of the drug on behavior and neurotransmitter release in the BF. Behavioral measures were simultaneously collected for an additional 1.5 h (total 12 h) post-microdialysis. All samples were collected at 4ºC and immediately stored at -80ºC until processed for ADO by HPLC/UV and AA/GABA by HPLC-EC detection.

Behavioral state analyses. Following completion of data collection, sleep-wakefulness was scored in 10 s epochs by examining the recordings visually using Neuroscore software (Data Sciences Inc., St Paul, MN). Any epochs that contained recording artifacts were tagged and excluded from subsequent analyses. EEG and EMG data were scored for waking (W), rapid eye movement (REM), and non-REM (NR) sleep. CBT and LMA (counts per minute) were analyzed as hourly means. Individual state data were analyzed as time spent in each state (W, REM, and NR) per hour. Latency to NR and REM onset for each rat was calculated from the time of drug injection. To assess any pharmacological effects on the consolidation of behavioral states, cumulative time spent in W, NR, and REM and the duration and number of bouts of each state was calculated for 3 h following drug administration relative to each 30 min dialysis sample obtained pre- and post-drug administration. Descriptive statistics and analysis of variance (ANOVA) analyses were performed on all behavioral measures. Where ANOVA indicated a probability (P) value < 0.05, Dunnett's *post hoc* was used to determine significance between groups.

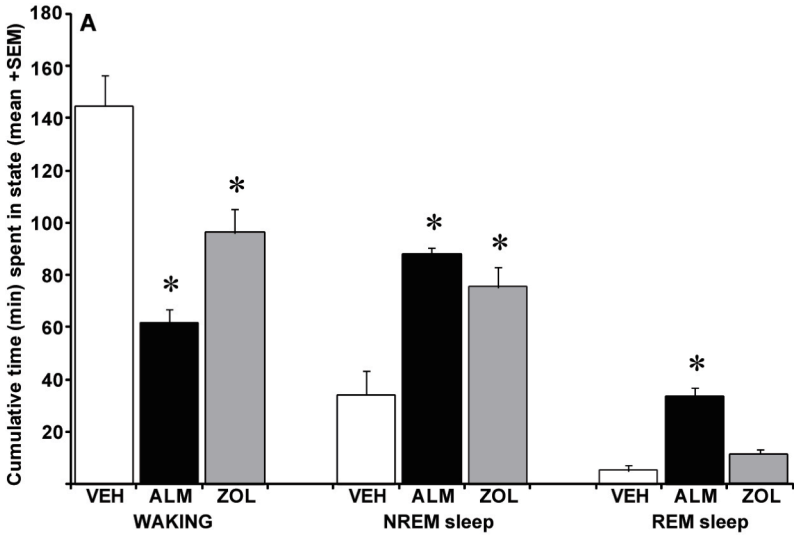

Figure 12. Time each behavioral state for VEH, ALM-, and ZOL-treated rats. Values (means ± SEM) are for a 3 h period during the dark phase. *p<0.05 for each treatment was determined by one-way ANOVA with Tukey's multiple comparison *post hoc* analyses.

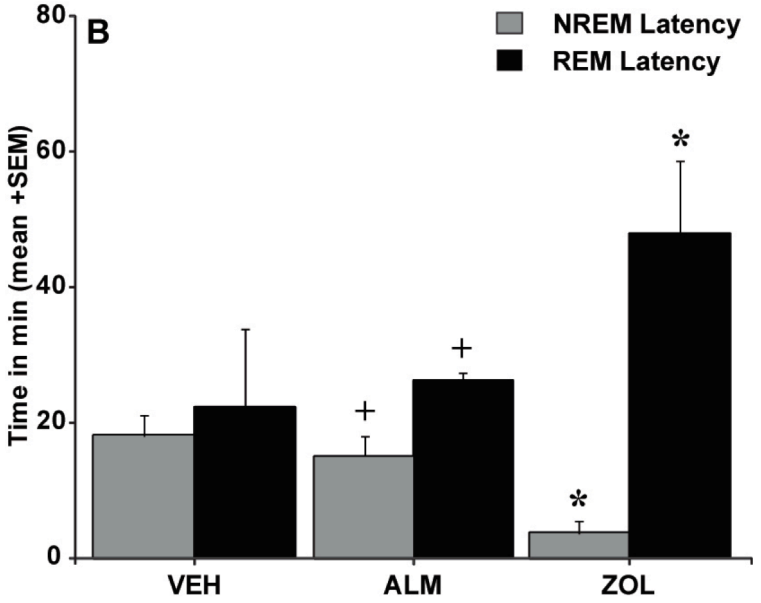

Figure 13. Latency to the onset of NREM and REM sleep following oral administration of ALM as compared to ZOL. * and + p<0.05 demonstrate significant differences from VEH (*) or from drug treatment (+).

HPLC Analyses. All microdialysis samples were split (10 µL for ADO, 20 µL for AA/GABA) into two vials for HPLC analyses. ADO samples were separated by reverse-phase HPLC with a Kinetic column (Phenomenex C18 150 x 4.6mm) and monitored at 254 nM by UV. The mobile phase consisted of 10 mM Na_2HPO_4 (pH = 4.5), and 7% acetonitrile and was set to a flow rate of 0.8 mL/min. Calibration curves were constructed using Chromeleon 6.8.0 software (Dionex, Corp). Amino acids, glutamate and GABA were assayed using HPLC-EC. The mobile phase consisted of 100

mM Na_2HPO_4, 22% MEOH, and 3.5% acetonitrile, pH 6.75 and set to a flow rate of 0.4 mL/min. The amino acids were detected by precolumn derivitization using O-phtaladehyde (OPA) and 2-mercaptoethanol (βME) with automation at 4°C, 2 min prior to injection into the HPLC. Separation was achieved with a reversed-phase column by Shiseido (Capcell Pak C18, 3.0 mm ID x 75 mm, 3 μm) and electrically detected at the following potentials; E1; +150 mV, E2; +550 mV, Guard +600 mV. Calibration curves were constructed using Chromeleon 6.8.0 software (Dionex Corp). Descriptive statistics and a two-way ANOVA were used to determine the effect of sleep-wake states on ADO, glutamate, and GABA release. Post hoc comparisons were performed using Tukey's multiple pairwise comparison tests. A probability (*P*) value < 0.05 was used to evaluate the significance of all statistical tests.

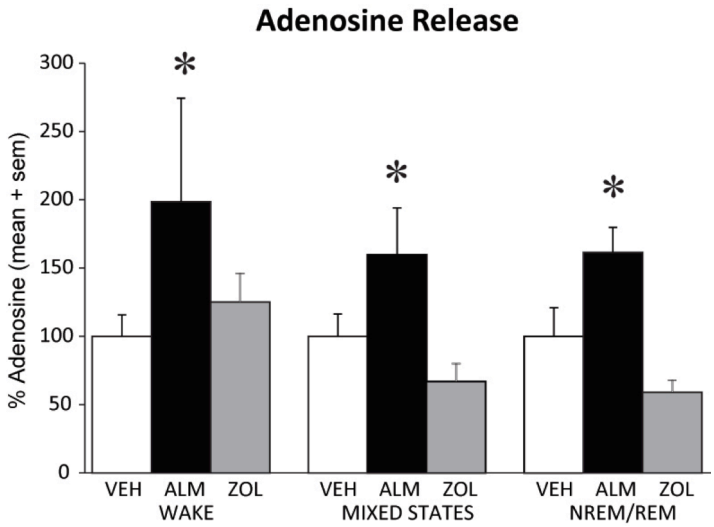

Figure 14. ADO release in the BF increased (*p<0.05) when sampled during all conditions following ALM (p.o.).

Results. To date, we have now completed recording, sampling and analysis of the proposed 8 rats per treatment group in Task 4B. Preliminary analyses were presented at the Society for Neuroscience meeting, Washington D.C. in November of 2011. As illustrated in Figure 12, the results of the effects of the various drugs on sleep-wake behavior demonstrate that ALM significantly promotes the amount of time spent in NREM and REM sleep as has been previously described (Dugovic et al., 2009). In addition, ALM significantly alters the latency to the onset of NREM and REM sleep compared to ZOL and VEH (Figure 13).

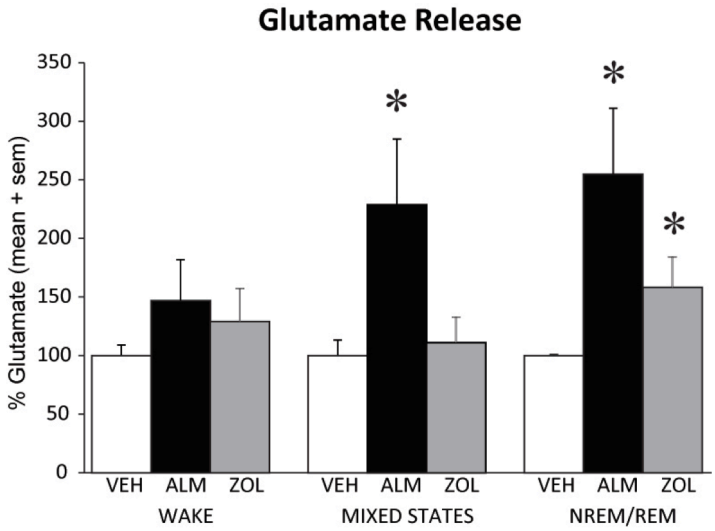

Figure 15. Glutamate release in the BF significantly increased after dosing with ALM when sampled during mixed states and NREM/REM compared to VEH (*p<0.05).

Dialysis samples were split into two and processed for both ADO and glutamate/GABA content. Two-way ANOVA revealed a significant drug x state interaction for all neurotransmitters. Tukey's *post hoc* comparisons (*p<0.05) were applied to determine significant differences between conditions.

The effects of the hypocretin antagonist ALM shows that this compound promotes adenosine (ADO) release in the basal forebrain (BF) during behavioral states of wakefulness, sleep, and mixed sleep-wake states (Figure 14). While ALM appears to promote ADO release in the BF during Wake, ZOL promotes

GABA release (Figure 16). During Mixed States, which involve transitions between Wake and Sleep, ALM also promotes GLU release (Figure 15). During predominantly NREM/REM sleep, both ALM and ZOL enhance GABA and GLU release (Figures 15 and 16).

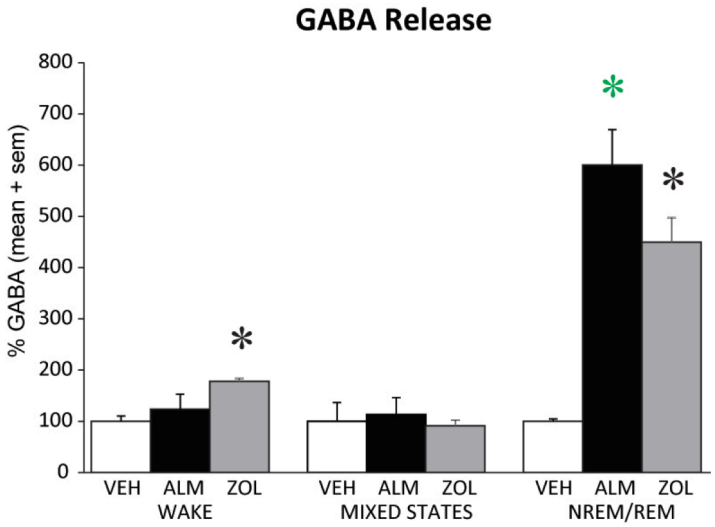

Figure 16. GABA release in the BF was significantly higher after dosing with ALM and ZOL during NREM/REM compared to VEH (*p<0.05).

As a result of positive feedback surrounding these data that were presented at the Society for Neuroscience, we decided to add an additional group of animals to the study aims to evaluate how ALM and ZOL's effects would alter neurotransmitter release under conditions of constant wakefulness.

Experimental design. Male Sprague-Dawley rats were implanted with chronic recording devices and cannulae directed at the BF as described above. Animals were given a 2-3 wk post-surgical recovery period prior to entering the experimental paradigm, conditioned to the microdialysis chamber, and handled for at least 30 min every day for 1 wk prior to the onset of the experiment to limit stress on the day of the experiment from exposure to a novel environment. A microdialysis probe was inserted through the guide cannula 16 h prior to the onset of the experiment day and continuously perfused with aCSF. We performed six hours of sleep deprivation (constant wakefulness) and permitted two hours of recovery sleep. At the start of the experiment (4.5 hours into the dark period, ZT16.5), four 30 min baseline samples (1 µL/min flow rate, 30 µL TV) were collected from freely-moving animals to assess basal levels of ADO, GLU, and GABA and baseline EEG, EMG, were collected to assess constant wakefulness. Each rat received one treatment in random order (washout period minimum 1 week) with parallel microdialysis sampling of the BF. Drug doses included ALM (100 mg/kg), ZOL (10 mg/kg) and VEH. One of three drugs was administered (p.o.) to the animals 6h into the dark period (ZT18), and nine 30 min samples were collected to assess the effects of the drug on constant wakefulness and neurotransmitter release in the BF. Additionally, four samples were collected for an additional two hours and the rats were allowed recovery sleep. All samples were collected at 4ºC and

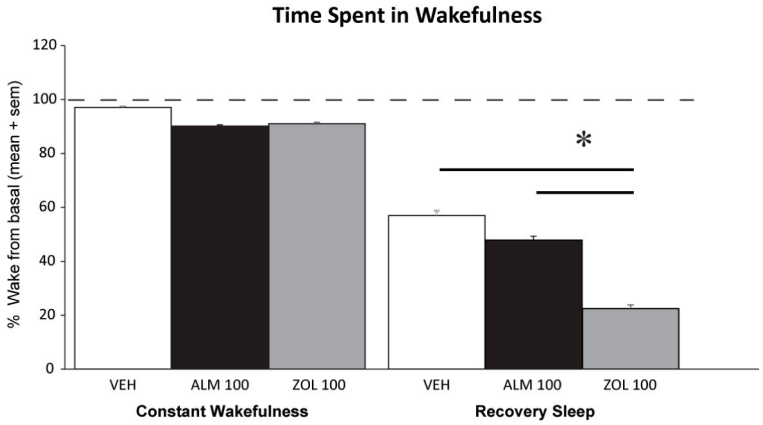

Figure 17. Percent time spent in wakefulness and recovery sleep following VEH, ZOL, and ALM treatment. *p<0.05 for each treatment was determined by one-way ANOVA with Tukey's multiple comparison *post hoc* analyses.

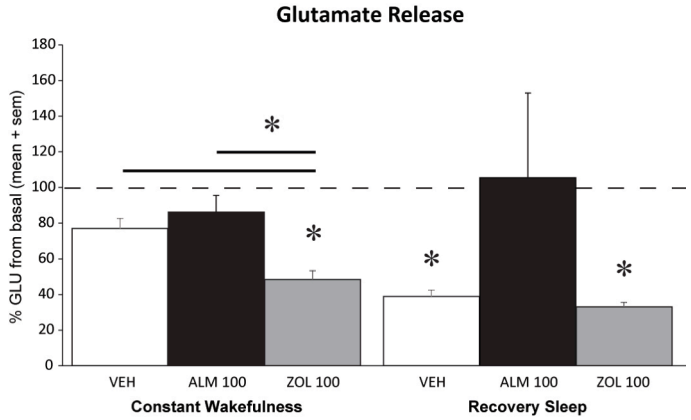

Figure 18. GLU release in the BF is not affected by ALM during waking and RS. ZOL caused a decrease in GLU release during wakefulness that persists into RS. *p<0.05 for each treatment was determined by one-way ANOVA with Tukey's multiple comparison *post hoc* analyses.

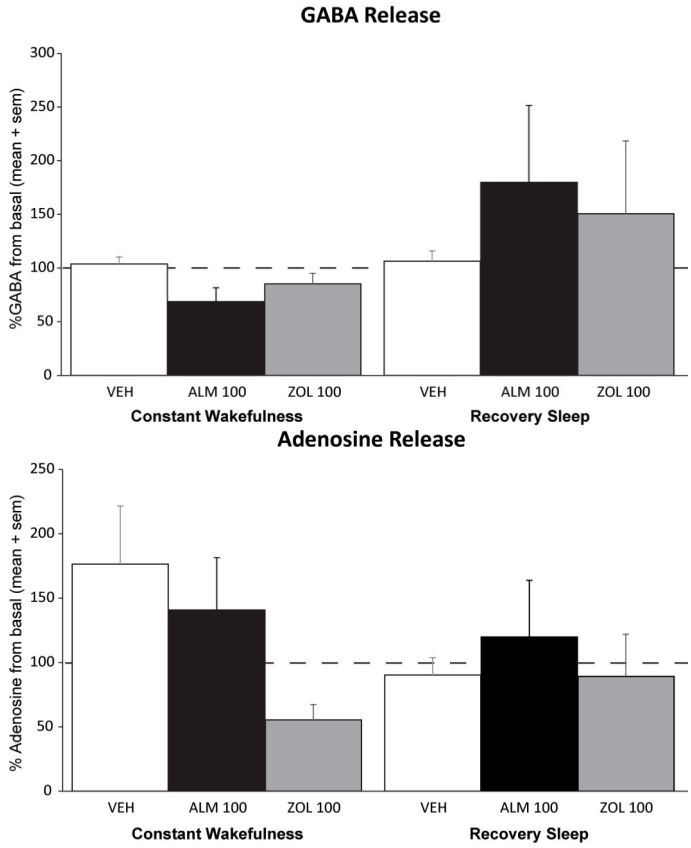

Figure 19. GABA and ADO release in the BF does not appear to be affected by either oral ALM or ZOL during constant waking or recovery sleep.

immediately stored at -80ºC until processed for ADO by HPLC/UV and AA/GABA by HPLC-EC detection.

Figure 17 summarizes the effects of VEH, ZOL, and ALM administration (p.o.) on constant wakefulness and recovery sleep for each treatment condition following drug delivery.

Analyses of neurotransmitter levels demonstrate GLU release in the BF is not altered by ALM when delivered during conditions of constant wakefulness or when the animals are permitted recovery sleep (*p<0.05; Tukey's *post hoc* test). However, oral ZOL significantly decreases BF GLU during constant waking that persists into recovery sleep (Figure 18; *p<0.05).

Preliminary results of GABA and ADO levels suggest that these neurotransmitter systems are not affected by drug administration under conditions of constant wakefulness and values appear to be near basal release levels upon recovery sleep (Figure 19). These neurotransmitter results provide additional evidence for dynamic neurochemical changes underlying Hcrt modulation of sleep-wakefulness.

All of these behavioral and neurotransmitter analyses will be submitted for publication once statistical power has been achieved with the appropriate number of animals per treatment group for constant wakefulness and recovery sleep. The originally proposed study design for Task 4C, BF ADO release in response ALM and ZOL by local dialysis, is not feasible as neither ALM nor ZOL readily pass across the dialysis membrane (testing in-house). Thus, we propose to continue evaluating ALM and ZOL effects on neurotransmitter levels in the BF under conditions of constant wakefulness and subsequent recovery sleep.

Task 6: *Utilize optogenetics and in vivo physiology to compare the neural circuitry underlying ALM-induced vs. ZOL-induced sleep.*

6a. Determine whether activation of the Hcrt system is sufficient to induce arousal in the presence of ALM vs. ZOL.

6b. Determine whether ALM affects the activity of subcortical sites downstream from the Hcrt neurons.

6c. Determine how ALM and ZOL affect the activity of cortical neurons.

Progress - Task 6: In order to achieve the goals proposed in Aim 6, it was necessary to build an *In Vivo* Cellular Neurophysiology Laboratory with the capabilities to perform the electrophysiology and optogenetic experiments proposed in Tasks 6a-c. Optogenetics experiments require that the animal under study expresses the Channelrodopsin-2 (ChR2) protein in the desired neuronal population, in our case, the HCRT neurons in the lateral hypothalamus. We chose the transgenic instead of the viral approach to achieve this, i.e., to breed a transgenic mouse line that expresses this light-sensitive ion channel only in HCRT neurons because it allows the manipulation of larger amounts of neurons and fortunately our collaborator in Japan, Dr. Akihiro Yamanaka has developed such a transgenic mouse and he provided us not only with the mice, but also he trained the postdoctoral fellow Dr. Jaime Heiss on the procedures to perform fiber optic implants in the lateral hypothalamus followed by tethered EEG/EMG recordings with optogenetic stimulation. Additionally, our existing *In Vitro* Cellular Neurophysiology Laboratory had to be upgraded in order to perform *in vitro* validation of the transgenic mice and optimization of the stimulation protocol by directly observing how single neurons are affected by the light stimulation.

Since the change in the Statement of Work and associated rebudgeting was approved in November, 2011, we have successfully built an *in vivo* lab that incorporates intracellular and

Table 1. Equipment acquired for the *In Vivo* Cellular Neurophysiology Laboratory

Instrument	Supplier	Model	Quantity
Vibration isolation table	TMC	63-544	1
Microelectrode amplifier	Molecular Devices	Multiclamp 700B	1
Data acquisition system	Molecular Devices	Digidata 1440	1
Light engine	Lumencor	Spectra 3	2
Oscilloscope	Hitachi	V-1565	1
Extracellular data acquisition system	Tucker-Davis Tech.	RZ2-4	1
96-channel preamplifier	Tucker-Davis Tech.	PZ2-96	1
Low impedance headstage	Tucker-Davis Tech.	RA16LI-D	1
Data streamer	Tucker-Davis Tech.	RS4-1	1
Implantable rodent headstage	Tucker-Davis Tech.	32-Channel ZIF-Clip	2
External battery	Tucker-Davis Tech.	PZ-BAT	1
Microwire array	Tucker-Davis Tech.	32 Channel ZIF-Clip	20
Microwire array	Tucker-Davis Tech.	16 Channel ZIF-Clip	20
4-axis micromanipulator	Siskiyou, Inc.	MX7600	2
Surgical microscope	Leica Microsystems	M320FL	1
Animal temperature controller	WPI, Inc.	ATC1000	1
Micro drill	WPI, Inc.	Omnidrill 35	1

extracellular recording systems with two computer-controlled light engines that enables us to successfully record intracellular and extracellular neuronal activity, local field potentials, EEG, and EMG while delivering light into the brain through an optical fiber. Table 1 provides a list of the equipment that has been installed in the *In Vivo* Cellular Neurophysiology Laboratory. This effort has also required an extensive amount of time to gain familiarity with the hardware and with the more than 10 different programs associated with it (including custom programing languages) and the inevitable fabrication of custom cables and miscellaneous other infrastructural adaptations. Figure 20 provides an example of one of our first recordings conducted in our new *In Vivo* Cellular Neurophysiology Laboratory from an awake mouse.

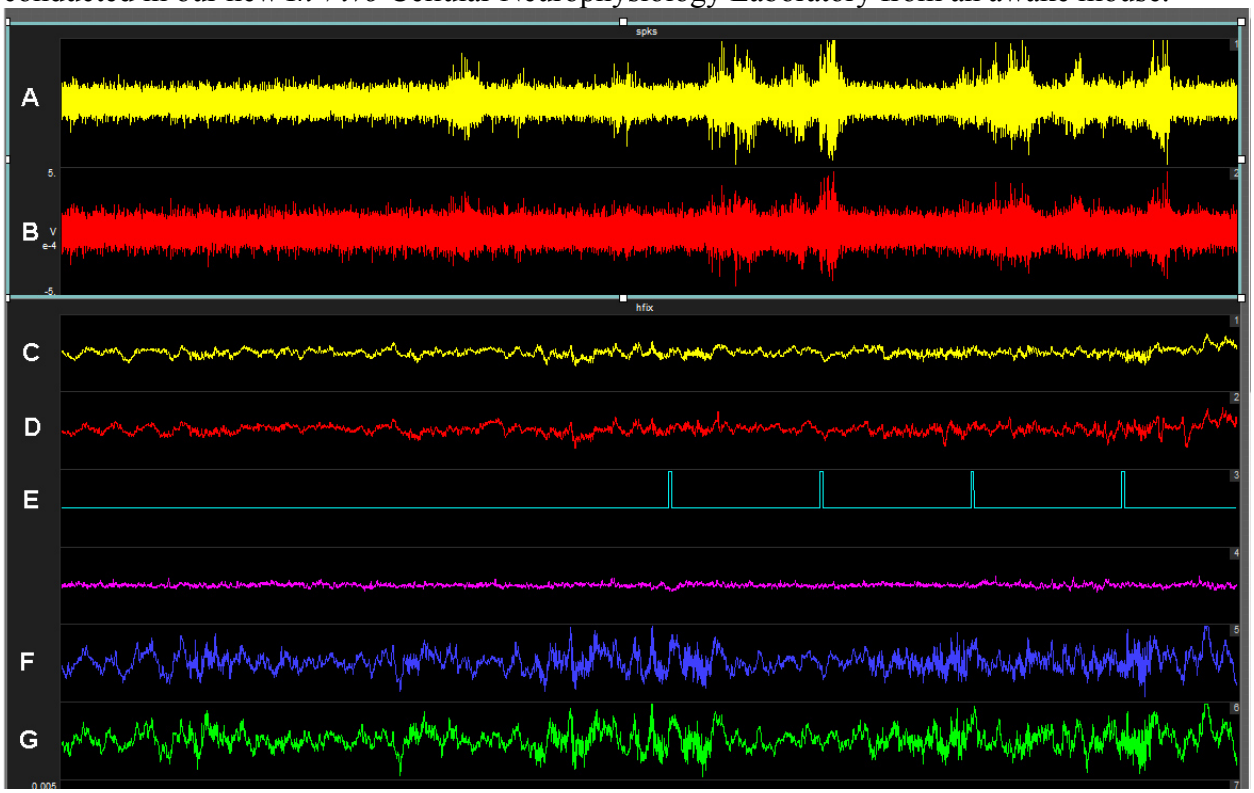

Figure 20. Screenshot of a 60 sec recording illustrating electrophysiological data acquisition using the TDT system. **A, B**: Cortical extracellular activity (MUA) from two different electrodes, **C,D**: EEG recordings from left parietal and right parietal electrodes, **E**: External stimulus signal, **F,G**: LFP traces recorded with the same two electrodes as in A and B.

In March, 2012, we received one breeding pair of *Orexin-tTA; Tet-O ChR2(C128S)* from our collaborator in Japan, Dr. Akihiro Yamanaka. Unfortunately, these mice were infected with *Helicobacter* spp. and thus required an extensive quarantine period upon arrival and they did not breed for more than two months after arrival at SRI International. Once litters were finally produced, we implemented a "cross fostering" protocol to eliminate the *Helicobacter* spp. from newborn pups. On 7/25/12, we received confirmation that some of the offspring are free of *Helicobacter* and can now join the mouse colony room to establish this transgenic line at SRI.

Plans for Year 4:

Task 2a: Analysis of the spatial reference memory study will be submitted for publication.

Task 2b: Data collection for the spatial working memory study will be completed. All data analysis will be completed and the results will be submitted for publication.

Task 2c: We anticipate we will complete the data collection and analysis for the rPVT study.

Task 3a: We will perform immunohistology on the processed tissue to quantify activation of wake-active cell populations defined by immunoreactivity for adenosine deaminase, serotonin, dopamine beta-hydroxylase, and choline acetyltransferase.

Task 3b: We will complete the remaining two lesion studies in Aim 3b, one of which (DBH-saporin lesions of the locus coeruleus) is currently in progress. Analysis of the basal forebrain lesion study will be submitted for publication, and data will be presented at the 2012 Society for Neuroscience meeting in New Orleans.

Task 3c: We anticipate receipt of the first of three transgenic mouse lines (Pet1-Lmx1b(ffp)) within the next several weeks, and we will initiate a breeding program to obtain an age-matched experimental cohort to be ready for study in early 2013. We are also expecting receipt of full cohorts of the remaining two strains (DBH-KO and Hdc-KO) this year, to be studied immediately upon arrival.

Task 4a: We will perform immunohistology on the processed tissue to quantify activation of sleep-active neurons in the ventro-lateral preoptic nucleus. Results on sleep-active cortical nNOS neurons will be presented at the 2012 Society for Neuroscience meeting in New Orleans.

Task 4b: In Year 4, we will continue collecting data from additional animals in order to reach our proposed statistical power of 8 rats per treatment group. The behavioral and neurotransmitter analyses will then be submitted for publication.

Task 6: Having now established the new laboratory and with a "clean" colony of *Orexin-tTA; Tet-O ChR2(C128S)* mice expected to be produced in Year 4, we will conduct Task 6a "Determine whether activation of the Hcrt system is sufficient to induce arousal in the presence of ALM vs. ZOL" as described in the revised Statement of Work.

KEY RESEARCH ACCOMPLISHMENTS

- Spatial reference memory study completed (Figures 1-4).

- Spatial working memory study under undisturbed conditions has been completed. All animals needed to complete the spatial working memory study under sleep-deprived conditions have been implanted and are currently under study.

- Equipment for the rPVT study (Task 2c) has been purchased.

- Publication of "Dual hypocretin receptor antagonism is more effective for sleep promotion than antagonism of either receptor alone" in *PLoS One* by Morairty et al.

- Completed rat perfusion and tissue processing for histological Tasks 3a and 4a.

- Completed the first of three lesion studies in Aim 3b, evaluating the efficacy of ALM vs. ZOL in basal-forebrain-lesioned or sham-operated animals (Figs 6-7).

- Successfully piloted a locus coeruleus lesion protocol for the second lesion study in Aim 3b (Fig. 8).

- Completed immunohistological analysis of neuronal activation of wake-active hypocretin neurons (Task 3a) and sleep-active cortical nNOS neurons (Task 4a).

- Presented poster entitled "The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake" at the Society for Neuroscience meeting held in Washington, D.C. in 2011 based on data collected in Tasks 2a, 3a and 4a.

- Presented poster entitled "Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat" at the Society for Neuroscience meeting held in Washington, D.C. in 2011 based on data collected in Task 4b.

- Completed the number of animals needed for Task 4b evaluating the effects of oral ALM vs. ZOL on neurotransmitter release in the Sprague-Dawley rat (Figs. 12-16)

- Have preliminary findings of the effects of oral ALM vs. ZOL on animals under conditions of constant wakefulness and recovery sleep (Figs. 17-19)

REPORTABLE OUTCOMES

Vazquez J., A. Nguyen, T. Kilduff. Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat. Program No. 720.09. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

Dittrich L, S. Morairty, A. Wilk, D. Warrier, K. Silveira, T.-M. Chen, T. S. Kilduff. The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake. Program No. 720.10. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

Morairty SR, Revel FG, Malherbe P, Moreau JL, Valladao D, Wettstein JG, Kilduff TS, Borroni E. Dual hypocretin receptor antagonism is more effective for sleep promotion than antagonism of either receptor alone. *PLoS One* 7(7):e39131. Epub 2012 Jul 2. PMID:22768296.

CONCLUSION

Results continued to accumulate that are consistent with the hypothesis that disfacilitation of wake-promoting systems by the hypocretin (Hcrt) receptor antagonist almorexant (ALM) results in less functional impairment than the inhibition of neural activity produced by the benzodiazepine receptor agonist zolpidem (ZOL). Preclinical data indicate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. Measures of both spatial reference memory (Task 2a) and spatial working memory (Task 2b) in

rodents treated with ALM were mostly indistinguishable from vehicle whereas impairments were clearly evident under ZOL. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. Similarly, wake-active Hcrt neurons can be recruited in the presence of ALM after sleep deprivation but not in the presence of ZOL (Task 3a). Conversely, although both drugs activate sleep-active cortical neurons, sleep-active cells are more strongly activated by ZOL (Task 4a). Lesions of the basal forebrain (BF), a wake-promoting area, potentiated the hypnotic effect of ZOL without affecting the response to ALM (Task 3b), indicating different neural pathways underlie the actions of these two drugs. ALM promoted adenosine and glutamate release in the BF (Task 4b) whereas ZOL promoted GABA release, particularly during waking. An *In Vivo* Cellular Neurophysiology Laboratory with the capabilities to perform the electrophysiology and optogenetic experiments (Tasks 6a-c) was established.

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Dugovic C, Shelton JE, Aluisio LE, Fraser IC, Jiang X, et al. (2009) Blockade of orexin-1 receptors attenuates orexin-2 receptor antagonism-induced sleep promotion in the rat. *J Pharmacol Exp Ther.* 330(1):142-51.

APPENDICES

1. Vazquez J., A. Nguyen, T. Kilduff. Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat. Program No. 720.09. *2011 Neuroscience Meeting Planner*. Washington, DC: Society for Neuroscience, 2011. Online.

2. Dittrich L, S. Morairty, A. Wilk, D. Warrier, K. Silveira, T.-M. Chen, T. S. Kilduff. The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake. Program No. 720.10. *2011 Neuroscience Meeting Planner*. Washington, DC: Society for Neuroscience, 2011. Online.

3. Morairty SR, Revel FG, Malherbe P, Moreau JL, Valladao D, Wettstein JG, Kilduff TS, Borroni E. Dual hypocretin receptor antagonism is more effective for sleep promotion than antagonism of either receptor alone. *PLoS One* 7(7):e39131. Epub 2012 Jul 2. PMID:22768296.

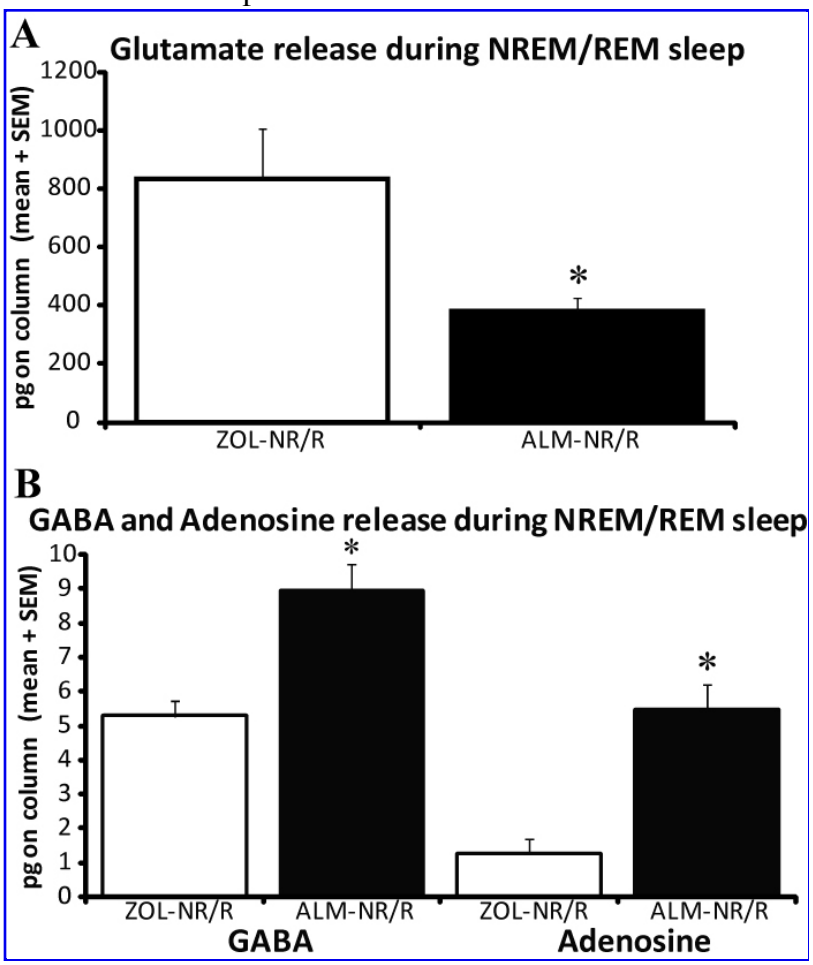
Presentation Abstract

Program#/Poster#:	720.09/XX21
Presentation Title:	Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat.
Location:	Hall A-C
Presentation time:	Tuesday, Nov 15, 2011, 1:00 PM - 2:00 PM
Authors:	***J. VAZQUEZ**, A. NGUYEN, T. KILDUFF; Ctr. for Neuroscience, Biosci. Div., SRI Intl., MENLO PARK, CA

Abstract:

Hypocretins (orexins) modulate diverse physiological processes such as cognitive function and alertness. Hypocretin-1 and hypocretin-2 (Hcrt) peptides regulate sleep and alertness (Kilduff and Peyron 2000) and Hcrt neurons project to several brain regions including the basal forebrain (BF; Peyron et al. 1998), a brain region critical for promoting wakefulness (Jones 2004). The BF contains cholinergic, GABAergic, and putative glutamatergic neurons important for cortical activation (Manns et al. 2003). Zolpidem (ZOL), a benzodiazepine receptor agonist, affects a Cl^- channel on the $GABA_A$ receptor, resulting in hyperpolarization and somnolence (Dang et al. 2010). In contrast, almorexant (ALM) is a dual Hcrt receptor antagonist that reversibly blocks signaling of both Hcrt peptides. Oral delivery of ALM elicits somnolence without cataplexy and, in rat, decreases active wake and increases the time spent in non-rapid eye movement (NREM) and (REM) sleep (Brisbare-Roch et al. 2007). We tested the hypothesis that oral ALM induces sleep by facilitating the mechanisms that underlie the transition to normal sleep. In contrast to ZOL, which affects $GABA_A$ receptors that are widely distributed in the CNS, ALM acts through blockade of post-synaptic Hcrt receptors, thereby disfacilitating excitation in the BF. We used in vivo microdialysis and HPLC analyses to examine BF glutamate, GABA, and adenosine efflux following oral ZOL (10mg/kg), ALM (100mg/kg), or placebo (VEH) combined with behavioral analyses. Two-way ANOVA revealed a significant drug x state interaction for all neurotransmitters.

Post-hoc comparisons showed that ALM (n=4 rats; $p<0.05$) caused a significant decrease in BF glutamate (**A**) during NREM/REM cycling and the corresponding collection timeframes compared to ZOL (n=4) or VEH (n=3; data not shown). Oral ALM concurrently increased BF GABA and adenosine (**B**; $p<0.05$) during NREM/REM compared to ZOL or VEH. These results provide novel evidence for dynamic neurochemical changes underlying Hcrt modulation of sleep-wakefulness.



Disclosures: **J. Vazquez:** None. **A. Nguyen:** None. **T. Kilduff:** None.

Keyword(s): MICRODIALYSIS

GABA

HYPOCRETIN

Support: USAMRMC W81XWH-09-2-0081

[Authors]. [Abstract Title]. Program No. XXX.XX. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

Presentation Abstract

Program#/Poster#:	720.10/XX22
Presentation Title:	The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake
Location:	Hall A-C
Presentation time:	Tuesday, Nov 15, 2011, 2:00 PM - 3:00 PM
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Abstract:	Most commonly prescribed hypnotics, such as benzodiazepine receptor agonists, cause general inhibition of neural activity. As a result, these hypnotics are less than optimal to aid sleep if there is a risk of being awakened with the need to perform without impairment, e.g., healthcare workers or emergency response personnel. A more specific mechanism of action is exerted by almorexant (ALM), a dual antagonist for hypocretin/orexin (Hcrt) receptors. We hypothesized that challenged rats would be able to stay awake more easily and function with less impairment after a sleep-promoting dose of ALM than after a dose of the benzodiazepine receptor agonist zolpidem (ZOL). To test this hypothesis, we trained 24 rats to remember the location of a platform in a spatial reference memory task (Morris Water Maze). Next day, they were dosed with either ALM (100 mg/kg i.p.), ZOL (30 mg/kg i.p.), or vehicle. Although both drugs induced sleep, the performance of rats dosed with ALM was indistinguishable from the rats dosed with vehicle whereas the group dosed with ZOL displayed weaker preference for the learned location of the platform. To assess the influence of the two compounds on the activity of sleep/wake regulatory neurons, we performed an immunohistological study using c-Fos as a marker of neuronal activity. The same rats were administered the drugs as described above but half of the animals were allowed to sleep for 1.5h after dosing, whereas the remaining rats were sleep deprived by gentle handling. In agreement with the behavioral results, we found that the percentage of

Fos-positive neurons in the wake-active Hcrt neurons in the lateral hypothalamus was higher for sleep deprived animals than for non-sleep deprived animals in the ALM and vehicle groups, whereas there was no such difference for the ZOL group. The sleep-active cortical neurons immunoreactive for neuronal nitric oxide synthase expressed more Fos in animals that were allowed to sleep than in those kept awake, independent from the compound administered. Taken together, our results indicate that ALM effectively induces sleep but unlike ZOL allows the rats to activate wake-promoting neurons and perform normally when needed.

Disclosures: **L. Dittrich:** None. **S. Morairty:** None. **A. Wilk:** None. **D. warrier:** None. **K. Silveira:** None. **T. chen:** None. **T.S. Kilduff:** None.

Keyword(s): WATER MAZE

C-FOS

SLEEP DEPRIVATION

Support: USAMRMC Grant W81XWH-09-2-0081

[Authors]. [Abstract Title]. Program No. XXX.XX. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

OPEN ACCESS Freely available online

PLoS one

Dual Hypocretin Receptor Antagonism Is More Effective for Sleep Promotion than Antagonism of Either Receptor Alone

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Abstract

The hypocretin (orexin) system is involved in sleep/wake regulation, and antagonists of both hypocretin receptor type 1 (HCRTR1) and/or HCRTR2 are considered to be potential hypnotic medications. It is currently unclear whether blockade of either or both receptors is more effective for promoting sleep with minimal side effects. Accordingly, we compared the properties of selective HCRTR1 (SB-408124 and SB-334867) and HCRTR2 (EMPA) antagonists with that of the dual HCRTR1/R2 antagonist almorexant in the rat. All 4 antagonists bound to their respective receptors with high affinity and selectivity *in vitro*. Since *in vivo* pharmacokinetic experiments revealed poor brain penetration for SB-408124, SB-334867 was selected for subsequent *in vivo* studies. When injected in the mid-active phase, SB-334867 produced small increases in rapid-eye-movement (REM) and non-REM (NR) sleep. EMPA produced a significant increase in NR only at the highest dose studied. In contrast, almorexant decreased NR latency and increased both NR and REM proportionally throughout the subsequent 6 h without rebound wakefulness. The increased NR was due to a greater number of NR bouts; NR bout duration was unchanged. At the highest dose tested (100 mg/kg), almorexant fragmented sleep architecture by increasing the number of waking and REM bouts. No evidence of cataplexy was observed. HCRTR1 occupancy by almorexant declined 4–6 h post-administration while HCRTR2 occupancy was still elevated after 12 h, revealing a complex relationship between occupancy of HCRT receptors and sleep promotion. We conclude that dual HCRTR1/R2 blockade is more effective in promoting sleep than blockade of either HCRTR alone. In contrast to GABA receptor agonists which induce sleep by generalized inhibition, HCRTR antagonists seem to facilitate sleep by reducing waking "drive".

Citation: Morairty SR, Revel FG, Malherbe P, Moreau J-L, Valladao D, et al. (2012) Dual Hypocretin Receptor Antagonism Is More Effective for Sleep Promotion than Antagonism of Either Receptor Alone. PLoS ONE 7(7): e39131. doi:10.1371/journal.pone.0039131

Editor: Roland Seifert, Medical School of Hannover, United States of America

Received February 13, 2012; **Accepted** May 16, 2012; **Published** July 2, 2012

Funding: Work at SRI International was supported in part by United States Army Medical Research and Materiel Command (USAMRMC) grant W81XWH-09-2-0081 (http://cdmrp.army.mil/default.shtml) and in part by institutional funds. Work at Roche was funded by Roche. USAMRMC had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Roche partly contributed to study design, data collection and analysis, decision to publish, and preparation of the manuscript.

Competing Interests: The authors have the following conflicts: Dr. Revel, Dr. Malherbe, Dr. Moreau, Dr. Wettstein, and Dr. Borroni are employed by Roche. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Determination of the functions of neurotransmitters, neuromodulators and their receptors has classically been aided by use of small molecule receptor-specific antagonists. In recent years, forward and reverse genetics have provided insights into the functions of neurotransmitter/neuromodulatory systems before receptor-specific antagonists were developed. Such was the case for hypocretin (orexin), whose cell bodies in the perifornical and lateral hypothalamus synthesize a pair of neuropeptides alternatively called hypocretin-1 (HCRT1) or orexin-A and hypocretin-2 (HCRT2) or orexin-B [1,2]. Identification of a mutation in the gene encoding HCRT receptor 2 (HCRTR2 or OX2R) as the cause of canine narcolepsy [3] and demonstration that HCRT ligand-deficient mice exhibited periods of behavioral arrest that resembled both human and canine narcolepsy [4] implicated the HCRT system in sleep/wake control well before the first small molecule HCRT receptor antagonists [5,6,7] were described. An extensive literature has since led to the conclusion that the HCRT system is wake-promoting [8,9,10,11] and involved in energy homeostasis [12,13]. Other studies have suggested roles for the HCRT system in neuroendocrine, cardiovascular, water balance, and gastrointestinal control [14], nociception and hyperalgesia [15,16,17], stress and stress-induced analgesia [18,19], reward and addiction [20,21,22,23], and panic anxiety [24].

It is currently unclear whether targeting the HCRTR2 alone or both HCRT receptors is the best strategy for the development of sleep-promoting compounds. Several dual HCRTR1/R2 antagonists show significant sleep-promoting properties [25,26,27,28,29,30,31,32]. However, some reports indicate that HCRTR2 blockade alone was sufficient to produce the hypnotic actions of HCRTR antagonism [32,33]. One study compared the efficacy of the selective HCRTR1 antagonist SB-408124 [34], the selective HCRTR2 antagonist JNJ-10397049 [35], and the dual

antagonist almorexant [27] and concluded that HCRTR1 antagonism attenuates the hypnotic actions of HCRTR2 blockade [32]. While data on the affinity and selectivity of these compounds have been published, the absence of information on their pharmacokinetic properties is problematic for interpretation of their *in vivo* effects.

In the present study, we characterize the hypnotic activity of HCRTR antagonists in rats to determine whether selective or dual HCRTR antagonists are more effective for promoting sleep. To ensure a meaningful *in vivo* comparison, we determined the pharmacological and pharmacokinetic profiles in rats of two selective HCRTR1 antagonists, SB-408124 and SB-334867 [36], the selective HCRTR2 antagonist EMPA [37], and the dual HCRTR1/R2 antagonist almorexant. After showing that SB-408124 displays insufficient brain penetration, we used SB-334867 as the HCRTR1 antagonist for all *in vivo* experiments. Lastly, we determined the time course of HCRTR occupancy by almorexant and correlated this with hypnotic efficacy.

Materials and Methods

Drugs

Almorexant (ACT-078573, (2*R*)-2-[(1*S*)-6,7-Dimethoxy-1-[2-(4-trifluoromethyl-phenyl)-ethyl]-3,4-dihydro-1H-isoquinolin-2-yl]-*N*-methyl-2-phenyl-acetamide) [27], EMPA *N*-(Ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulfonyl)-amino]-*N*-pyridin-3-yl-methyl-acetamide) [37], SB-674042 (1-(5-(2-fluoro-phenyl)-2-methyl-thiazol-4-yl)-1-((S)-2-(5-phenyl-(1,3,4)oxadiazol-2-yl-methyl)-pyrrolidin-1-yl)-methanone) [34], and Cp-5 ((*S*)-1-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-3,3-dimethyl-2-[(pyridin-4-ylmethyl)-amino]-butan-1-one) [7] were synthesized at F. Hoffmann-La Roche Ltd. (Basel, Switzerland) or SRI International (Menlo Park, CA USA) according to the patent literature [38]. SB-334867 (1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl-urea hydrochloride), zolpidem (N,N,6-Trimehtyl-2-(methylphenyl)-imidazol[1,2-a]pyridine-3-acetamide) and SB-408124 (1-(6,8-difluoro-2-methyl-quinolin-4-yl)-3-(4-dimethylamino-phenyl)-urea) were purchased from Tocris Bioscience (Ellisville, MO). Chemical structures are provided in Figure S1. [^{3}H]almorexant (specific activity: 42.7 Ci/mmol), [^{3}H]SB-674042 (specific activity: 24.4 Ci/mmol) and [^{3}H]EMPA (specific activity: 94.3 Ci/mmol) were synthesized at Roche.

Animals

Animal experiments performed at F. Hoffmann-La Roche were conducted in strict adherence to the Swiss federal regulations on animal protection and to the rules of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and with the explicit approval of the local Cantonal Veterinary Office/Authority Basel City. Animal experiments performed at SRI International were approved by SRI's Institutional Animal Care and Use Committee and were in accordance with U.S. National Institute of Health guidelines. Male Wistar rats (240±20 g) used for spontaneous locomotion studies and pharmacokinetic studies at F. Hoffmann-La Roche were obtained from RCC Ltd. (Fullinsdorf, Switzerland). Male Sprague-Dawley rats (300±25 g) used for receptor occupancy studies at F. Hoffmann-La Roche were from Iffa Credo (Lyon, France). Animals were housed in separate rooms under a 12 h light/12 h dark cycle (light onset: 06:00, except where noted below; Zeitgeber time 0, ZT0) at 22±2°C, with *ad libitum* access to food and water. Male Sprague-Dawley rats (300±25 g) used for sleep studies at SRI were from Charles River (Wilmington, MA) and were housed in a temperature-controlled recording room under a 12 h light/12 h dark cycle (lights on at 05:00) with food and water available *ad libitum*. Room temperature (24±2°C), humidity (50±20% relative humidity), and lighting conditions were monitored continuously via computer. Animals were inspected daily in accordance with AAALAC and SRI guidelines.

Pharmacological Studies

[^{3}H]almorexant binding to rat HCRTR1 and HCRTR2. The rat cDNAs encoding HCRTR1 (Accession No. P56718) and HCRTR2 (Accession No. P56719) were subcloned into pCI-Neo expression vectors (Promega, Madison, WI) and used to transfect HEK293 cells (acquired commercially from ATCC-LGC, Molsheim, France) as previously described [37]. Membrane preparations, saturation and inhibition experiments, and determination of the association and dissociation kinetic parameters of [^{3}H]almorexant to rHCRTR2-HEK293 cell membranes were performed at F. Hoffmann-La Roche as previously described [37] and reported in the Materials and Methods S1.

Pharmacokinetic Studies

Pharmacokinetic analyses were performed at F. Hoffmann-La Roche as described in supporting Maqterials and Methods S1.

SB-334867 selectivity screen. SB-334867 was evaluated in a selectivity screen performed at CEREP (Paris, France). The screen consisted of binding assays on a panel of 79 target receptors. The specific binding (SB) of a radioligand to each target receptor was defined as the difference between the total binding and the nonspecific binding determined in the presence of a cold competitor in excess. The results are expressed as a percent of control SB obtained in the presence of SB-334867 used at 10 μM. Details on the CEREP screen are available from www.cerep.fr.

Effect of Almorexant and SB-334867 on Spontaneous Locomotor Activity in Rats

Locomotor activity (LMA) was evaluated at F. Hoffmann-La Roche as described previously [39]. Male Wistar rats were placed for 2 weeks in a 12 h light/12 h dark cycle with light onset at 22:00 (ZT0). Three h after the onset of the dark period (i.e., ZT15), rats were injected ip with either vehicle or HCRT receptor antagonist (almorexant or SB-334867 at 3, 10, 30 mg/kg in NaCl 0.9%, 0.3% Tween-80) (n = 8 per group), and returned to the recording chambers. Spontaneous LMA was recorded for a period of 30 min. At the end of the experiment, the brain and plasma were collected for determination of the drug exposure and brain/plasma concentration ratio.

Electroencephalogram, Core Body Temperature and Locomotor Activity Studies

Surgical procedures and recordings. All rodent electroencephalograph (EEG) studies were performed at SRI International. Three groups of eight male Sprague-Dawley rats (300±25 g; Charles River, Wilmington, MA) were implanted with chronic recording devices (F40-EET, Data Sciences Inc., St Paul, MN) for continuous recordings of EEG, electromyograph (EMG), core body temperature (T_{core}), and LMA via telemetry as previously described [40]. Data recording and scoring were performed as previously reported [40] (see also Supplemental Material and Methods). The EEG and EMG data were scored in 10 sec epochs for waking (W), rapid eye movement sleep (REM), and non-REM sleep (NR). T_{core} and LMA (counts per minute) were analyzed as hourly means. Data from the EEG studies are

reported in hourly means such that the hourly time ZT1 refers to the hour between time points ZT0 and ZT1.

Experimental design. For each of the three separate studies, a repeated measures counter-balanced design was employed in which each rat received five separate dosings. The dosing conditions for study 1 included SB-334867 at three concentrations (3, 10 and 30 mg/kg), zolpidem (ZOL, 7.5 mg/kg) and a vehicle control (saline 95%/ethanol 5%). The dosing conditions for study 2 included EMPA at three concentrations (10, 30 and100 mg/kg), ZOL (10 mg/kg) and a vehicle control (HPMC). The dosing conditions for study 3 included almorexant at three concentrations (10, 30 and 100 mg/kg), ZOL (10 mg/kg) and a vehicle control (HPMC). All dosings were administered ip in a volume of 2 ml/kg. A minimum of 3 d elapsed between doses. Dosing occurred during the middle of the rats' normal active period at the start of ZT19 and was typically completed within 10 min. Animals were continuously recorded for 6 h prior to dosing and for 18 h following dosing.

Determination of HCRTR1 and HCRTR2 occupancy by almorexant. This study was performed at F. Hoffmann-La Roche. Sixty-five male Sprague-Dawley rats, housed 5 per cage (light onset: 12:00), were injected intraperitoneally (ip) with either vehicle (1.25% hydroxypropyl methylcellulose (HPMC), 0.1% docusate sodium) or almorexant (30 mg/kg in 1.25% HPMC, 0.1% docusate sodium) at the mid-dark phase (ZT18; i.e., 6 h after lights-off), and returned to their home cage. Groups (n = 5 per group) of vehicle- or almorexant-treated animals were then sacrificed by decapitation 0.5, 2, 4, 8 or 12 h after the injection. An extra group of non-injected rats (n = 5) was also sampled at ZT18. Plasma was collected and stored at −80°C until assayed. Brains were rapidly dissected, frozen on dry ice, and stored at −80°C. Series of coronal brain sections (14 µm) were cut in a cryostat through the posterior hypothalamus (tuberomammillary nucleus level: 3.8 to 4.2 mm posterior to bregma) and the brain stem (dorsal raphe nucleus level: 7.3 to 8 mm posterior to bregma; locus coeruleus level: −9.3 to −10 mm posterior to bregma), thaw-mounted (6 sections per slide) and stored at −20°C. After sectioning, the remaining pieces of brain were kept at −80°C for later determination of almorexant brain concentration. The brain and plasma concentrations of almorexant were determined by quantitative liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS).

Receptor occupancy (RO) was determined as published previously [41]. For each Hcrt receptor subtype, two series of slides were thawed and incubated at room temperature with the relevant radioligand in assay buffer for 15 min (HCRTR1) or 1 h (HCRTR2). For HCRTR1, assay buffer (2 mM $CaCl_2$, 5 mM $MgCl_2$, 25 mM HEPES, pH 7.4, 100 µL per section) contained either 5 nM [^{3}H]SB-674042 (for determination of total binding, TB) or 5 nM [^{3}H]SB-674042 plus 10 µM SB-408124 (for determination of non-specific binding, NSB). For HCRTR2, assay buffer (1 mM $CaCl_2$, 5 mM $MgCl_2$, 25 mM HEPES, pH 7.4, 120 µL per section) contained either 1 nM [^{3}H]EMPA (for determination of TB) or 1 nM [^{3}H]EMPA plus 10 µM Cp-5 (for determination of NSB). The liquid was drained, the brain sections were rinsed with ice-cold assay buffer (2 brief washes followed by 3×2 min soaking) and distilled water (3 brief dips) and air dried at 4°C for 12 h. The slides were exposed together with [^{3}H] microscales against tritium-sensitive imaging plates (BAS-TR2025) for 5 days. The plates were scanned with a high resolution phosphor imager device (Fujifilm BAS-5000) and calibrated measurements of radioactivity (fmol/mg protein) were made. All analyses were performed blind to treatment.

For each selected region, the mean signal density (TB) was measured and averaged from three consecutive sections from the same slide. The specific binding (SB) signal was then determined for each animal by subtracting the NSB signal from the TB signal. NSB was measured from adjacent brains sections incubated with the radiotracer and an excess of cold competitor. The SB signal was averaged for each experimental group and the percent RO was calculated at each time-point according to the equation $RO = (1-(SB_{almorexant}/SB_{vehicle}))\times 100$, where $SB_{almorexant}$ is the average SB for the animal group injected with almorexant and $SB_{vehicle}$ is the average SB for the animal group injected with vehicle.

Statistical Analyses

Results are shown as mean±SEM. LMA and RO data were analyzed with one-way ANOVA followed by Dunnett's analysis. EEG data were analyzed with repeated measures (rm)-ANOVA, followed by paired two-tailed *t*-tests. REM:NR ratios, sleep latencies (NR and REM) and cumulative data were analyzed with one-way rm-ANOVA and all other data with two-way rm-ANOVA. Light period and dark period data were analyzed separately as well as pre- and post-drug administration data. Statistical significance was set at $P<0.05$.

Results

Pharmacological Studies

Binding characteristics of [^{3}H]almorexant to rHCRTR1- and rHCRTR2-expressing cell membranes. To characterize the *in vitro* binding of [^{3}H]almorexant to rat HCRT receptors, saturation binding analyses were performed at binding equilibrium on membranes isolated from HEK293 cells transiently transfected with rHCRTR1 and rHCRTR2. As shown in Fig. 1A and B, [^{3}H]almorexant bound with high affinity to a single saturable site on recombinant rHCRTR1 (K_d of 3.4±0.3 nM and B_{max} of 27.2±0.7 pmol/mg prot, at 23°C) and rHCRTR2 (K_d of 0.5±0.0 nM and B_{max} of 53.0±1.4 pmol/mg prot, measured at 37°C). Binding kinetics of [^{3}H]almorexant to membrane preparations from HEK293 cells transiently expressing rHCRTR2 are shown in Fig. 1C and D and the kinetic parameters in Table 1. The association binding of [^{3}H]almorexant to the rHCRTR2 had a half-maximal binding at 10 min and reached equilibrium within 50 min. The data were fit to a one-phase exponential model with the association rate constant of 0.073±0.015 $nM^{-1}min^{-1}$. The dissociation rate for [^{3}H]almorexant binding to the rHCRTR2 was determined by the addition of an excess amount of almorexant (5 µM) after equilibrium was reached. The rate of [^{3}H]almorexant dissociation from rHCRTR2 membrane was slow; the reversal of binding was incomplete and did not reach baseline even after 2 h (Fig. 1D & Table 1).

The potencies of almorexant and of the selective HCRTR1 antagonists SB-334867 [6] and SB-408124 [34] in inhibiting [^{3}H]almorexant binding to HEK293-rHCRTR1 and HEK293-rHCRTR2 cell membranes are given in Table 2. Almorexant was able to displace [^{3}H]almorexant binding from rHCRTR1 and rHCRTR2 membranes with high affinity (Table 2). In contrast, SB-334867 and SB-408124 displaced [^{3}H]almorexant binding from rHCRTR1, but not from rHCRTR2, with high affinity (Table 2).

Pharmacokinetic Studies

Pharmacokinetic properties of SB-334867, SB-408124, EMPA and almorexant in rats. The oral bioavailability and pharmacokinetic properties of almorexant, SB-334867 and SB-408124 were evaluated in Wistar rats. The mean pharmacokinetic parameters after single iv or oral (po) bolus administration in rat

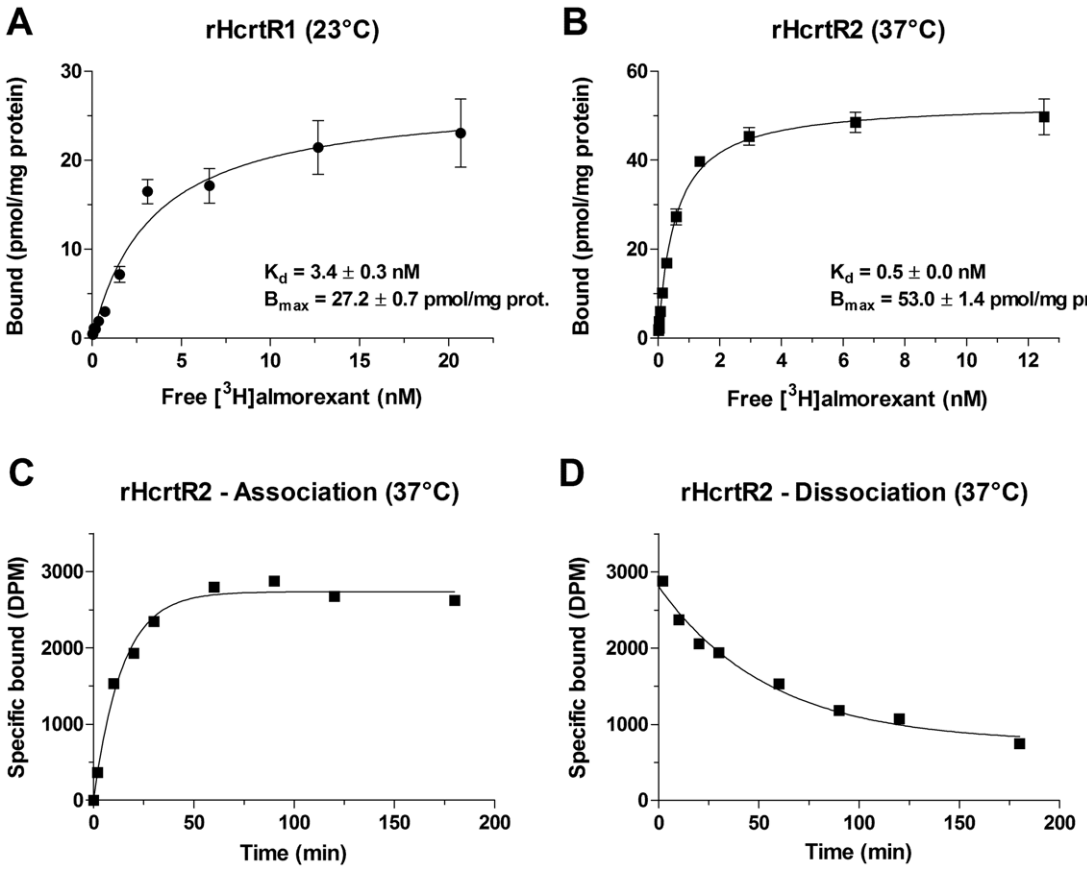

Figure 1. Binding characteristics of [^{3}H]almorexant to rHCRTR1 and rHCRTR2 cell membranes. (**A,B**) Saturation binding curves of [^{3}H]almorexant binding to membranes from HEK293 cells transiently transfected with rHCRTR1 (**A**) or rHCRTR2 (**B**). Each data point represents the mean±SEM of three independent experiments performed in triplicate. The data were analyzed by nonlinear regression analysis using GraphPad Prism 4.0 software and a single-site binding model. (**C,D**) Time course for the association (**C**) and dissociation (**D**) of [^{3}H]almorexant binding to rHCRTR2 membranes.
doi:10.1371/journal.pone.0039131.g001

are given in Table S1. Almorexant displayed a high systemic plasma clearance, high volume of distribution at steady state (Vss) and low oral bioavailability in rat. In addition, almorexant was highly bound to plasma proteins (<3.7%, and <8.7% free fraction in human and rat plasma, respectively), and its stability measured for 2 h in human and rat plasma was 90.0% and 95.0%, respectively. The mean brain/plasma concentration ratio of almorexant was 0.12 in rat.

SB-334867 exhibited a low systemic plasma clearance, medium Vss and oral bioavailability in rat. SB-334867 is highly bound to plasma proteins (1.3%, and 0.8% free fraction in human and rat plasma, respectively), and its stability measured for 1 h/4 h in human and rat plasma was 95%/93% and 104%/110%, respectively. The mean brain/plasma concentration ratio of SB-334867 (at a dose of 8.8 mg/kg, po) was 0.53 in rat.

SB-408124 had a low systemic plasma clearance, low Vss and medium oral bioavailability in rat. SB-408124 had very low free fraction in human and rat (0.3% and <0.1%, respectively) and its stability (1 h/4 h) in human and rat plasma was 94%/88% and 101%/107%, respectively. The mean brain/plasma concentration ratio of SB-408124 (at dose of 18 mg/kg, po) was 0.03 in rat. Such unfavorable pharmacokinetic properties of SB-408124, most importantly its extremely low brain penetration, prompted us to use SB-334867 for further *in vivo* studies in the rat.

The pharmacokinetic profiles of EMPA have been reported previously [37].

Table 1. Kinetic parameters for the association and dissociation of [^{3}H]almorexant in rHCRTR2-HEK293 cell membranes at 37°C.

Compound	Association kinetic	Dissociation kinetic	Apparent	
	K_{on} ($nM^{-1}min^{-1}$)	K_{off} (min^{-1})	$t_{1/2}$ (min)	K_d (nM)
[^{3}H]almorexant	0.073±0.015	0.021±0.004	36.3±5.7	0.33±0.9

The K_{on} (calculated on rate), K_{off} (observed off rate), $t_{1/2}$ (half-maximal binding) and K_d (apparent dissociation constant) values are ± SEM, calculated from three independent experiments (each performed in quadruplicate) as described under ''Materials and Methods''.
doi:10.1371/journal.pone.0039131.t001

Table 2. Potencies of almorexant, SB-408124 and SB-334867 antagonists in inhibition of [^{3}H]almorexant binding to the membrane preparations from HEK293 cells transiently expressing rHCRTR1 and rHCRTR2.

Compound	rHCRTR1	rHCRTR2
	[^{3}H]almorexant (23°C)	[^{3}H]almorexant (37°C)
	K_i (nM)	K_i (nM)
almorexant	7.1±0.7	2.0±0.0
SB-408124	45.7±4.1	5370.0±2200.0
SB-334867	58.4±2.9	2390.0±81.0

[^{3}H]almorexant was used at a concentration equal to its K_d values of 3.4 nM and 0.5 nM at rHcrtR1 and rHcrtR2, respectively, in these competition binding experiments. K_i values for [^{3}H]almorexant binding inhibition by various antagonists were calculated as described under "Materials and Methods". Values are ± SEM of the K_i calculated from three independent experiments, each performed in duplicate.

doi:10.1371/journal.pone.0039131.t002

Selectivity profile of SB-334867. The specificity of SB-334867 at the HCRTR1 was confirmed by assessment in radioligand binding assays in a broad CEREP screen (Paris, France; www.cerep.fr) (Table S2). Among the 79 receptors tested, 30 were peptide receptors. SB-334867 was inactive (<40% activity at 10 μM) at all targets tested with the exception of the A_{2A} (adenosine), A_3, MT3 (melatonin), P_{2Y} (purinergic 2Y) and $5HT_{2C}$ (serotonin 2C) receptors, where it caused 89%, 63%, 102%, 64% and 70% displacement of specific binding at 10 μM, respectively. The selectivity profiles of almorexant [27] and EMPA [37] have been reported previously.

Effect of Almorexant and SB-334867 on Spontaneous Locomotor Activity in Rats

The ability of almorexant and SB-334867 to antagonize *in vivo* the biological action of endogenous hypocretins was assessed by measuring spontaneous LMA during the active phase. Almorexant dose-dependently reduced LMA, although only the 30 mg/kg dose reached significance when compared to vehicle (Figure 2A; $F = 4.28$, $p<0.05$). Similarly, SB-334867 dose-dependently reduced spontaneous LMA, with both the 10 and 30 mg/kg doses being statistically different from vehicle (Figure 2B; vehicle: 6097±536; 10 mg/kg: 3509±383; 30 mg/kg: 2626±341; $F = 12.80$, $p<0.01$ and $p<0.001$, respectively).

The plasma and brain exposure of SB-334867 were measured at the end of the LMA experiment. When determined 35 min after ip administration, SB-334867 doses of 3, 10 and 30 mg/kg produced plasma levels of 220, 718 and 738 ng/mL vs. brain levels of 48, 171, and 142 ng/mL (ratios: 0.21, 0.23, 0.19, respectively). These results confirmed the ability of SB-334867 to enter the rat brain at the doses used in this report.

Rodent EEG Studies

The effects of almorexant, SB-334867 and EMPA administered in the middle of the dark (active) period were evaluated during the latter half of the active period and subsequent light (inactive) period to determine both efficacy for sleep promotion and whether "hangover" or rebound effects occurred. Of these three compounds, only almorexant reduced NR and REM sleep latency (Figure 3). Almorexant at 30 and 100 mg/kg reduced NR latency while only the 30 mg/kg concentration decreased latency to REM sleep. ZOL produced a decrease in NR latency in all three experiments.

In contrast, all three compounds increased NR sleep (Figure 4). SB-334867 at 3 and 30 mg/kg increased cumulative NR for the first 4 and 6 h periods following administration ($F = 10.808$, $p<0.0001$ and $F = 10.752$, $p<0.0001$, respectively). EMPA at 100 mg/kg also increased cumulative NR for the first 4 and 6 h periods post administration ($F = 17.655$, $p<0.0001$ and $F = 12.816$, $p<0.0001$, respectively). Almorexant had the strongest effect: both 30 and 100 mg/kg increased cumulative NR for 2, 4 and 6 h following administration ($F = 13.010$, $p<0.0001$; $F = 17.771$, $p<0.0001$; and $F = 16.179$, $p<0.0001$, respectively). Cumulative REM also increased for the first 2 h following almorexant at 30 mg/kg ($F = 5.418$, $p = 0.0023$) and for the 6 h period following the 100 mg/kg dose (Figure 4; $F = 8.535$, $p<0.0001$). ZOL increased cumulative NR and decreased cumulative REM in all three experiments. Whereas ZOL suppressed the REM:NR ratio in all 3 studies, none of the 3 test compounds did (Table 3). Although ZOL had significant effects on EEG delta power during NR, this parameter was little affected by any of the three test compounds compared to vehicle control (Figure S2).

There were few effects on sleep/wake amounts during the light period subsequent to administration of EMPA, SB-334867 or almorexant (Figure 5). REM was not significantly affected during this period following any of the three HCRT antagonists. NR decreased during the third hour of the light period (ZT3) following SB-334867 at 10 and 30 mg/kg while NR increased during ZT1 and ZT6 following almorexant at 30 mg/kg compared to vehicle. No significant effects on NR were found following EMPA during the light period.

Significant results occurred in measures of sleep-wake consolidation (Tables S3, S4, S5 and Figures S3, S4, S5). The strongest effects were found following almorexant at 100 mg/kg, which produced increased numbers of W and NR bouts during ZT19, ZT20, and ZT22-ZT24 ($F = 2.069$, $p = 0.0077$ and $F = 2.413$, $P = 0.0015$, respectively). The number of REM bouts was increased by almorexant at 100 mg/kg during ZT22-ZT24 ($F = 2.963$, $p = 0.002$). W bout duration was decreased following almorexant at 100 mg/kg during ZT22 compared to vehicle ($F = 2.320$, $p = 0.0023$). All three concentrations of EMPA increased the number of W bouts ($F = 4.243$, $p = 0.0065$). SB-334867 increased NR bout duration during ZT21 following 30 mg/kg and during ZT24 following 3 mg/kg ($F = 4.574$, $p<0.0001$).

Both LMA and T_{core} underwent dose-dependent decreases after drug treatment (Figure 6). ANOVA revealed condition effects for both almorexant and EMPA in which LMA was decreased across the 6 h period following administration of both compounds at 100 mg/kg compared to vehicle ($F = 7.316$, $p<0.00015$ and $F = 7.442$, $p = 0.00018$ respectively). No differences in LMA during the subsequent light period were found. Condition effects for T_{core} were found in all three studies. The high concentrations tested for all three HCRT receptor antagonists decreased T_{core} across the 6 h period following administration ($F = 7.629$, $p = 0.00027$ for SB-334867; $F = 7.442$, $p = 0.00018$ for EMPA; $F = 7.315$, $p = 0.00036$ for almorexant). ZOL administration resulted in the largest declines in T_{core} in all three studies, which was followed by a sustained rebound increase in T_{core} during the subsequent light period.

Time Course of HCRT Receptor Occupancy (RO) by Almorexant

To determine the time-course of HCRTR1 and HCRTR2 RO by almorexant, a single dose of almorexant at the smallest concentration shown to promote sleep (30 mg/kg, ip; Figure 4) was administered in the mid-dark phase (ZT18) and rats were

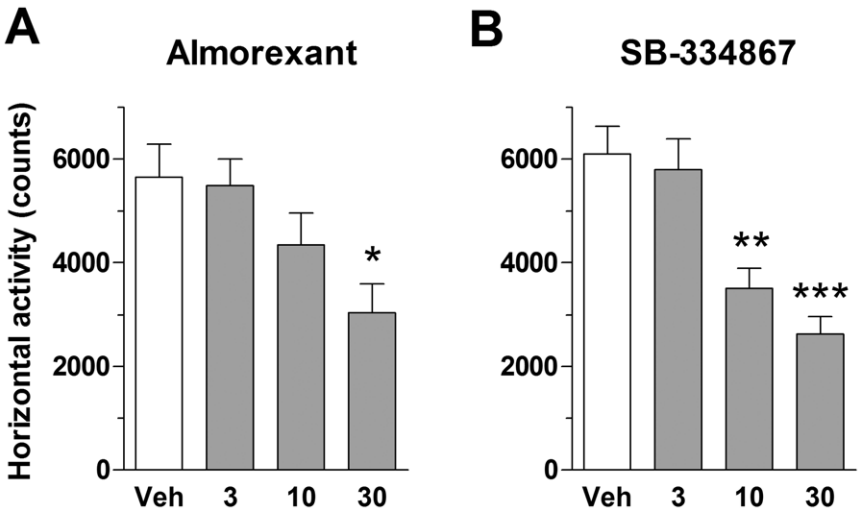

Figure 2. Effects of almorexant and SB-334867 on spontaneous locomotor activity of rats during the active phase. Both almorexant **(A)** and SB-334867 **(B)** reduced locomotor activity compared to vehicle (Veh) when administered 3 h after the onset of the dark period. Horizontal locomotor activity was recorded for a period of 30 min. Numbers on the X-axes represent intraperitoneal doses in mg/kg.***$p<0.001$, **$p<0.01$, *$p<0.05$ vs. Veh (one-way ANOVA followed by Dunnett's analysis). All data are mean±SEM (n = 8 per group).
doi:10.1371/journal.pone.0039131.g002

sacrificed after incubation periods of 0, 0.5, 2, 4, 8 or 12 h. For both HCRTR1 and HCRTR2, the NSB was minimal and represented 6.2% and 3%, respectively, of the average TB signal measured in control animals. The signal localization was in good agreement with the distribution of *HCRTR1*- and *HCRTR2*-expressing neurons [42,43], as confirmed by *in situ* hybridization on separate sections (data not shown). Figure 7A shows representative autoradiograms of HCRTR1 binding sites in the locus coeruleus (*LC*). This signal localization is in good agreement with the distribution of *Hcrtr1*-expressing neurons [42,43], as confirmed by *in situ* hybridization (data not shown). The rats injected with vehicle displayed maximal HCRTR1 radiotracer binding at all time points (Figure 7A), whereas the animals injected with almorexant showed reduced binding 2 h after the injection. Binding of the HCRTR1 radiotracer returned to levels similar to control 8–12 h post almorexant injection.

Figure 7B shows representative autoradiograms of the HCRTR2 binding sites examined at 2 different rostro-caudal levels. At the level of the posterior hypothalamus, signal was observed in various brain regions, including the tuberomammillary nuclei (*TMN*), cerebral cortex (*CC*), retrosplenial cortex (*RSC*), and field CA3 of the hippocampus (*CA3*). The signal attributed to the *TMN* was verified by *in situ* hybridization for histidine decarboxylase mRNA on separate sections (data not shown). At the level of the anterior pons, the dorsal raphe nuclei (*DRN*), pontine nuclei (*Pn*) and parabigeminal nuclei (*PBG*) displayed specific labeling. This pattern corresponds to that already reported by Malherbe et al. [37] and was in good agreement with the distribution of *Hcrtr2*-expressing neurons previously described [42,43]. The rats injected with vehicle displayed constant HCRTR2 binding at all time points. In contrast, the animals that received almorexant exhibited a very strong reduction of HCRTR2 radiotracer binding and, 2 h after almorexant injection, no signal could be detected (Figure 7B). Reduction of TB signal was still evident for all brain regions 12 h after almorexant administration.

SB was quantified in the *LC* for HCRTR1 and in 6 brain areas (*TMN*, *CC*, *CA3*, *RSC*, *DRN* and *Pn*) for HCRTR2, and the RO by almorexant was determined for 12 h post-injection (Figure 7C and Figure S7). HCRTR1 RO reached 50–60% from 30 min to 4 h post-injection (maximum: 59% after 2 h) and then returned to basal levels after 6 h. This RO profile paralleled that of almorexant concentration in the plasma (Figure 7D) and brain (Figure S6). For both compartments, drug concentration rose rapidly and reached a peak around 30 min, with plasma levels of 1966.4±349.2 ng/mL and brain levels of 565.8±112.4 ng/g (mean brain/plasma concentration ratio: 0.28). The half-maximal concentrations were achieved between 4 and 6 h.

For HCRTR2, all 6 structures displayed a comparable RO profile (Figure 7C for *DRN* and *TMN*, and Figure S7 for *CC*, *RSC*, *Pn* and *CA3*): it was close to 100% within 30 min after dosing, remained at maximal levels at 2 h and 4 h, and started to slowly decline between 4 and 6 h. After 12 h, although the brain and plasma levels of almorexant were strongly reduced (Figure 7C and Figure S6), HCRTR2 occupancy was still elevated with levels between 49 and 67%, depending on brain structure (Figure 7C and Figure S7; *TMN*: 49.2±13.2%; *CC*: 66.1±11.6%; *CA3*:58.4±11.5%; *RSC*: 64.6±10.7%; *DRN*: 57.7±10.5%; *Pn*: 67.2±13.9%).

Discussion

This study was undertaken to determine whether blockade of either or both HCRT receptors is more effective in promoting sleep. Multiple dual HCRTR1/R2 antagonists employing different molecular scaffolds have been found to have significant significant sleep-promoting properties [25,26,27,28,29,30,31] Anatomical localization of HCRTRs suggests that both receptors are involved in the promotion of wakefulness [39,43]. High levels of HCRTR1 are found in *LC* while only HCRTR2 is abundant in the *TMN*. Both receptors are expressed at moderately high levels in the dorsal and medial raphe and in the cholinergic regions of the basal forebrain. In the laterodorsal tegmentum and the pedunculopontine nucleus (brain stem cholinergic regions), the HCRTR1 is predominant. However, some recent reports support the hypothesis that only blockade of the HCRTR2 underlies the hypnotic actions of HCRTR antagonism [30,31]. Further, one study suggests that antagonism

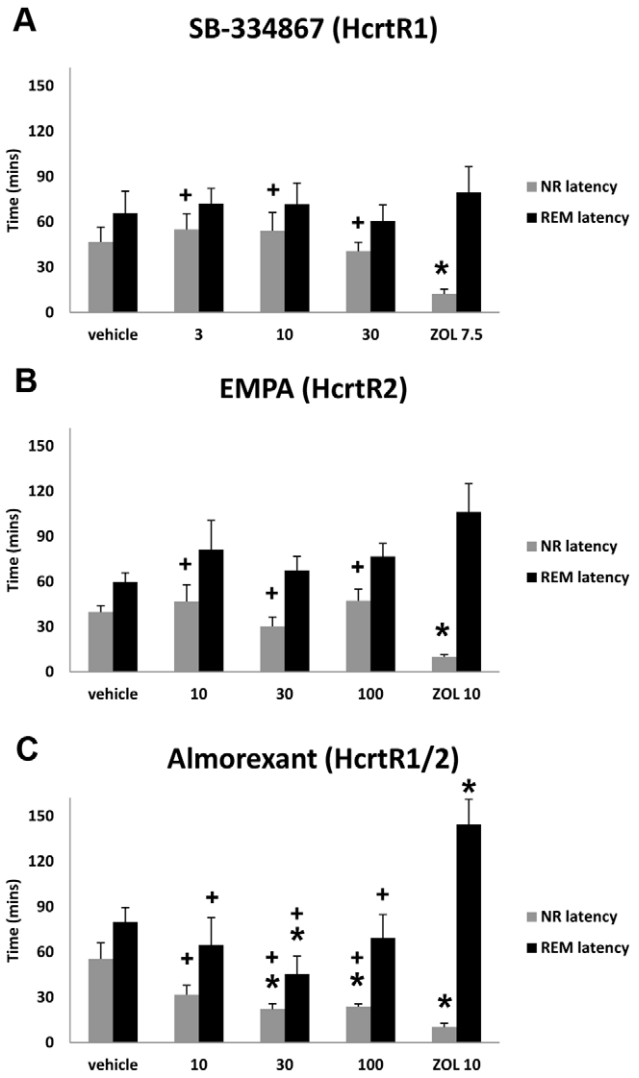

Figure 3. Latency to the onset of NR and REM sleep following administration of SB-334867. (A), EMPA **(B)**, and almorexant **(C)** as compared to zolpidem (ZOL). *=significantly different from vehicle ($p<0.05$); +=significantly different from ZOL ($p<0.05$) (One-way repeated measures ANOVA followed by paired two-tail *t* tests; n=8 per group). Data represent the mean±SEM.
doi:10.1371/journal.pone.0039131.g003

of HCRTR1 attenuates the hypnotic actions of HCRTR2 blockade [32]. Therefore, to help clarify the hypnotic effects of HCRTR blockade, we characterized the pharmacological and pharmacokinetic properties of selective and dual HCRTR antagonists in rat before evaluating their relative efficacy on sleep and wakefulness.

Pharmacokinetic Considerations

The affinities of almorexant, SB-408124 and SB-334867 at the rat HCRTR1 and HCRTR2 receptors are very similar to those reported for human HCRT receptors (for almorexant, K_i values of 4.7 nM and 0.9 nM at hHCRTR1 and hHCRTR2, 37°C, respectively [42]; for SB-334867, K_i value of 38.7 nM at rHCRTR1 [34]; for SB-408124, K_i value of 26.9 nM at rHCRTR1 [34]). Almorexant had high affinity for both HCRTRs and displayed a slow rate of dissociation from rHCRTR2 membranes *in vitro*, which translated into a long-lasting occupancy of the HCRTR2 *in vivo*. This property likely underlies some of the pharmacological effects described here. Among the three antagonists tested, almorexant had the highest systemic plasma clearance, highest Vss but lowest oral bioavailability; both SB-334867 and SB-408124 had low clearances and medium to low bioavailability. Importantly, SB-408124 had a very low free fraction and was found to penetrate the brain poorly, especially when compared to the other compounds. This prompted us to use SB-334867 for evaluating the effects of selective HCRTR1 blockade on sleep.

Effects of Selective HCRTR1 and HCRTR2 Antagonists on Sleep/wake

Selective blockade of HCRTR2 clearly results in sleep promotion. The HCRTR2 antagonist JNJ-10397049 reduced NR latency during both the light and dark phases, increased NR duration in the light phase, and increased both NR and REM duration during the dark phase [30,31]. Here, although EMPA had no effect on either NR or REM latency when administered in the mid-dark phase, it increased cumulative NR for the first 4 and 6 h. Conversely, icv infusion of an HCRTR2 agonist, [Ala^{11}]orexin-B, during the light period dose-dependently increased wake duration and decreased the amounts of both NR and REM sleep [44]. The effects of HCRT1 (orexin-A) on wakefulness and NREM sleep were reduced more in $OX2R^{-/-}$ mice than in $OX1R^{-/-}$ mice, implying that HCRTR2 has a greater influence than HCRTR1 on these parameters, at least in mice [45].

The selective HCRTR1 antagonist SB-334867 dose-dependently reduced LMA and, at 3 and 30 mg/kg i.p., increased cumulative NR for the first 4 and 6 h. These results differ from those of Dugovic *et al.* [32] who reported that selective blockade of HCRTR1 using SB-408124 had no effect on sleep, although it reduced LMA. However, the time of drug administration differed between these studies (middle vs. start of the active phase). By the middle of the active phase, both endogenous HCRT tone [46,47] and sleep pressure are increased, so HCRTR antagonists are more likely to be effective at this time of day than at dark onset.

A previous study showed that SB-334867 blocked HCRT1-induced effects on REM sleep but did not alter any sleep parameters when administered alone [36]. However, only the first hour after treatment was examined whereas, here, effects of SB-334867 on sleep were only apparent after 2 h. Importantly, we showed that SB-408124 exhibits poor pharmacokinetic properties, with notably low free fraction and little brain penetration, which likely limits its *in vivo* efficacy. The brain-to-plasma ratio for SB-408124 is 0.03, which is in the range of blood contamination levels obtained with the residual blood carried over in the brain homogenate (in the absence of compound in the brain). Although Dugovic *et al.* [32] did not specifically report brain-to-plasma ratios, they did report both brain and plasma concentrations following administration of SB-408124 at 30 mg/kg. Using these numbers, a brain-to-plasma ratio for SB-408124 is calculated to be 0.012 (using C_{max} values given in text: brain-to-plasma ratio = 1.09/84.29 = 0.012), which is in good agreement with our findings. This observation most likely explains why Dugovic *et al.* [32] did not detect effect on sleep. There are numerous examples of compounds lacking central efficacy due to insufficient brain exposure. For example, the reduced ability of second-generation H1 anti-histaminic drugs to cross the blood-brain barrier (BBB) as compared to the first generation of drugs, prevents them from causing centrally-mediated side effects such as sedation [48,49,50]. Similarly, the antidiarrheal medication loperamide is a potent agonist of the μ opiate receptor that is devoid of opioid central effects at usual doses in patients [51]. This directly results from the

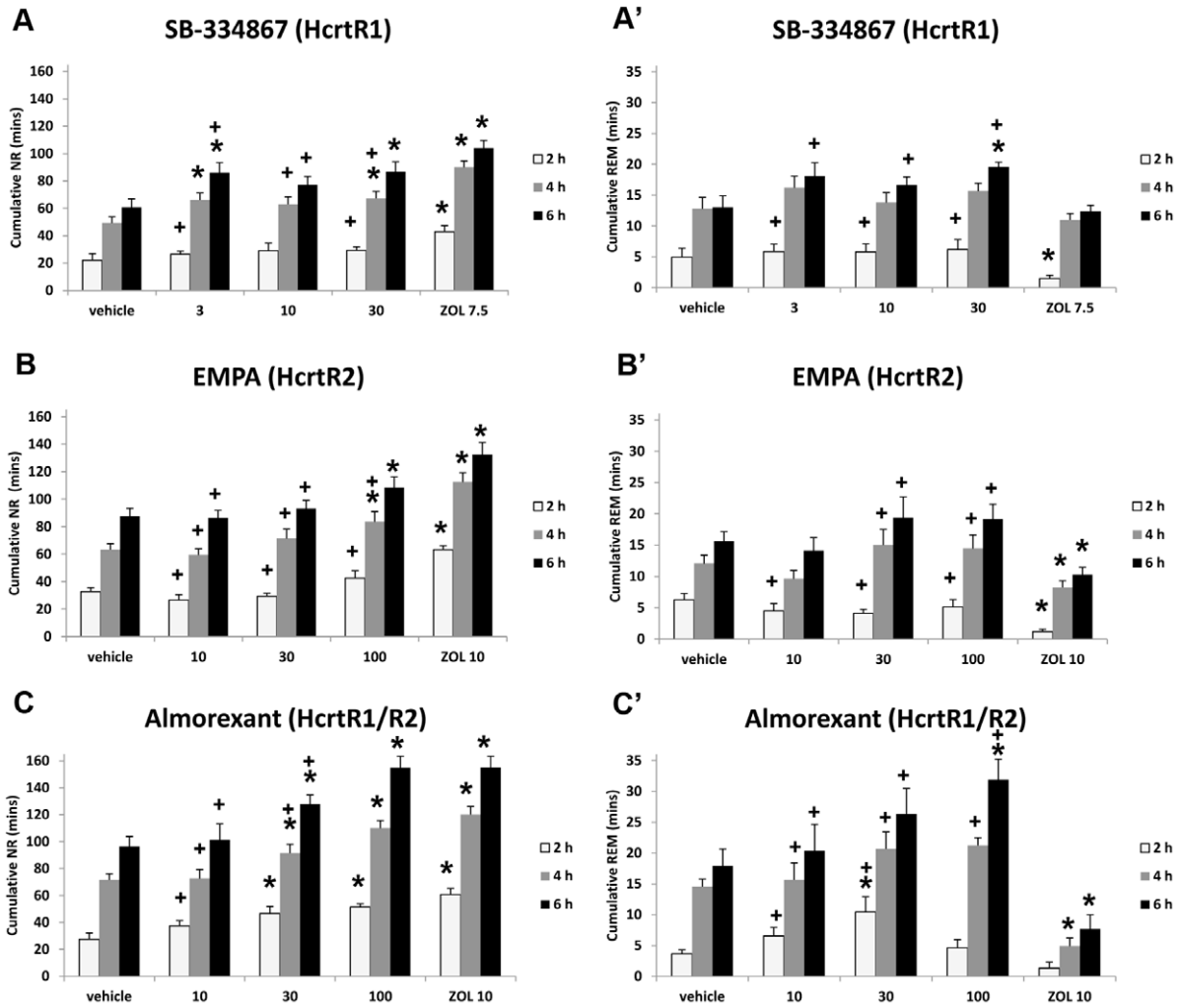

Figure 4. Cumulative time in NR and REM sleep over the first 2, 4 and 6 h following drug administration. (A–C) Cumulative time spent in NR sleep following SB-334867 (**A**), EMPA (**B**) and almorexant **(C)** compared to zolpidem (ZOL). **(A'–C')** Cumulative time spent in REM sleep for the same drug treatments. (One-way repeated measures ANOVA followed by paired two-tail *t* tests; n = 8 per group). Data represent the mean±SEM. *, significantly different from vehicle; **+**, significantly different from ZOL.
doi:10.1371/journal.pone.0039131.g004

Table 3. REM:NR ratios for the 6 h period following the administration of SB-334867, EMPA and almorexant.

Vehicle	**SB-334867**	**SB-334867**	**SB-334867**	**ZOL**
	3 mg/kg	**10 mg/kg**	**30 mg/kg**	**7.5 mg/kg**
0.22±0.039	0.21±0.016^{+}	0.22±0.023^{+}	0.23±0.016^{+}	0.12±0.009*
Vehicle	**EMPA**	**EMPA**	**EMPA**	**ZOL**
	10 mg/kg	**30 mg/kg**	**100 mg/kg**	**10 mg/kg**
0.18±0.018	0.16±0.019^{+}	0.21±0.032^{+}	0.18±0.027^{+}	0.08±0.008*
vehicle	**Almorexant**	**Almorexant**	**Almorexant**	**ZOL**
	10 mg/kg	**30 mg/kg**	**100 mg/kg**	**10 mg/kg**
0.18±0.020	0.19±0.026^{+}	0.20±0.022^{+}	0.21±0.021^{+}	0.05±0.013*

* = significantly different from vehicle ($p<0.05$), $^{+}$ = significantly different from ZOL ($p<0.05$).
doi:10.1371/journal.pone.0039131.t003

low brain exposure caused by the P-glycoprotein (P-gp) transporter at the BBB [51]. Administration of the drug to P-gp-deficient mice or co-administration with a P-gp blocker both increase brain levels and trigger central effects typically observed with brain penetrant opioids, such as analgesia [52,53] or respiratory depression [54]. Our observation made with SB-408124 underscores that verification of brain penetration is a prerequisite for the conception and use of centrally-acting drugs [55,56].

On the other hand, it is difficult to reconcile the poor brain penetration of SB-408124, both documented here and also evident in the study of Dugovic *et al.* (estimation: 0.012), with some indications of central localization following subcutaneous administration of 30 mg/kg, i.e. the 90% HCRTR1 occupancy observed in the *tenia tecta* and the SB-408124-mediated elevation of extracellular dopamine levels in the prefrontal cortex [32]. A heterogeneous distribution of the drug is unlikely, and further experiments will be necessary to delineate more precisely the free concentration of the compound, such as microdialysis studies and measures of binding to brain tissue homogenates.

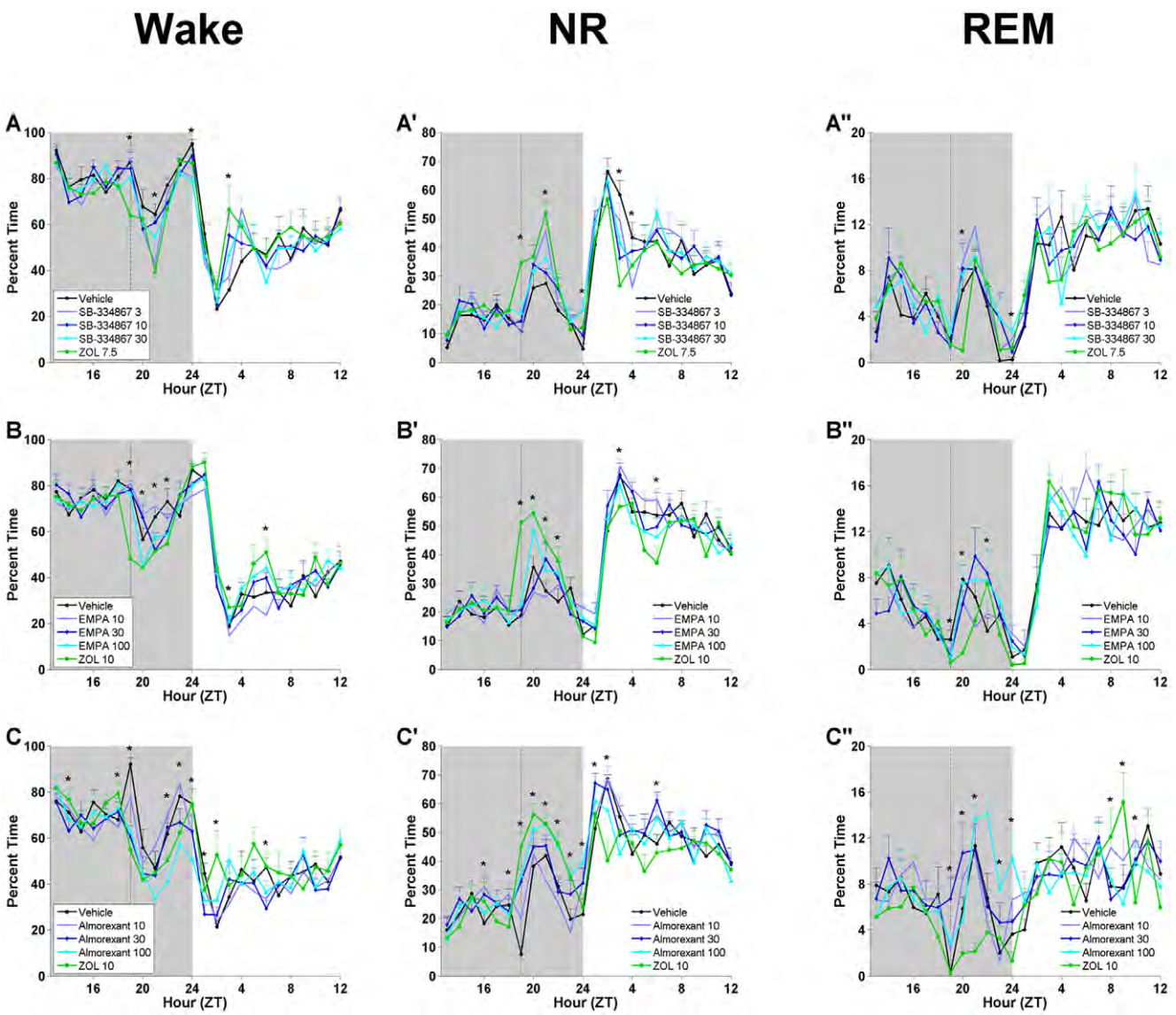

Figure 5. Hourly distribution of W, NR and REM sleep. W, NR and REM sleep for 6 h prior to and 18 h after administration of SB-334867 **(A)**, EMPA **(B)**, and almorexant **(C)** as compared to zolpidem (ZOL) and vehicle. Shaded area represents the dark phase; vertical dotted line in each panel indicates the time of injection. **(A)** Hourly amounts of wakefulness following SB 334867. **(A')** Hourly amounts of NR sleep following SB 334867. **(A'')** Hourly amounts of REM sleep following SB 334867. **(B)** Hourly amounts of wakefulness following EMPA. **(B')** Hourly amounts of NR sleep following EMPA. **(B'')** Hourly amounts of REM sleep following EMPA. **(C)** Hourly amounts of wakefulness following almorexant. **(C')** Hourly amounts of NR sleep following almorexant. **(C'')** Hourly amounts of REM sleep following almorexant. Data represent the mean±SEM (n = 8 rats per group). *, $p<0.05$. For detailed statistical results, see Text S1.
doi:10.1371/journal.pone.0039131.g005

Dual HCRTR Antagonists as Potential Hypnotic Medications

Dual HCRTR1/R2 antagonists are now well-established to induce sleep. In rats, almorexant administered po at the beginning of the dark phase promoted both NR and REM sleep and, at a higher dose, reduced NR and REM latency [27]. The effects on sleep duration but not sleep latency were confirmed when almorexant was administered sc [32]. Here, we report that almorexant given ip at the mid-dark phase also increases sleep duration. However, in contrast to Dugovic *et al.*, we found that almorexant at 30 and 100 mg/kg reduced NR latency and the 30 mg/kg dose also decreased REM latency. These differences likely reflect the greater sensitivity of the sleep/wake bioassay when injections occur in the mid-dark period after a sleep debt has accumulated. Recently, other dual HCRTR1/R2 antagonists have also been reported to reduce active wake and increase both NR or delta sleep and REM sleep when administered near the mid-dark phase [25,26,27,28,29,30,31,57]. Thus, multiple HCRTR1/R2 antagonists seem to be effective in inducing sleep.

Our results indicate some promising aspects of HCRT antagonists as hypnotic agents. First, in contrast to current hypnotics such as zolpidem which increase NR and suppress REM sleep, none of the three HCRTR antagonists affected the REM:NR ratio, indicating that both REM and NR increased proportionally. Second, in comparison to zolpidem, HCRTR antagonists only triggered a limited, physiological reduction of body temperature. Lastly, no excess wakefulness was observed during the subsequent light period. A proportional increase of

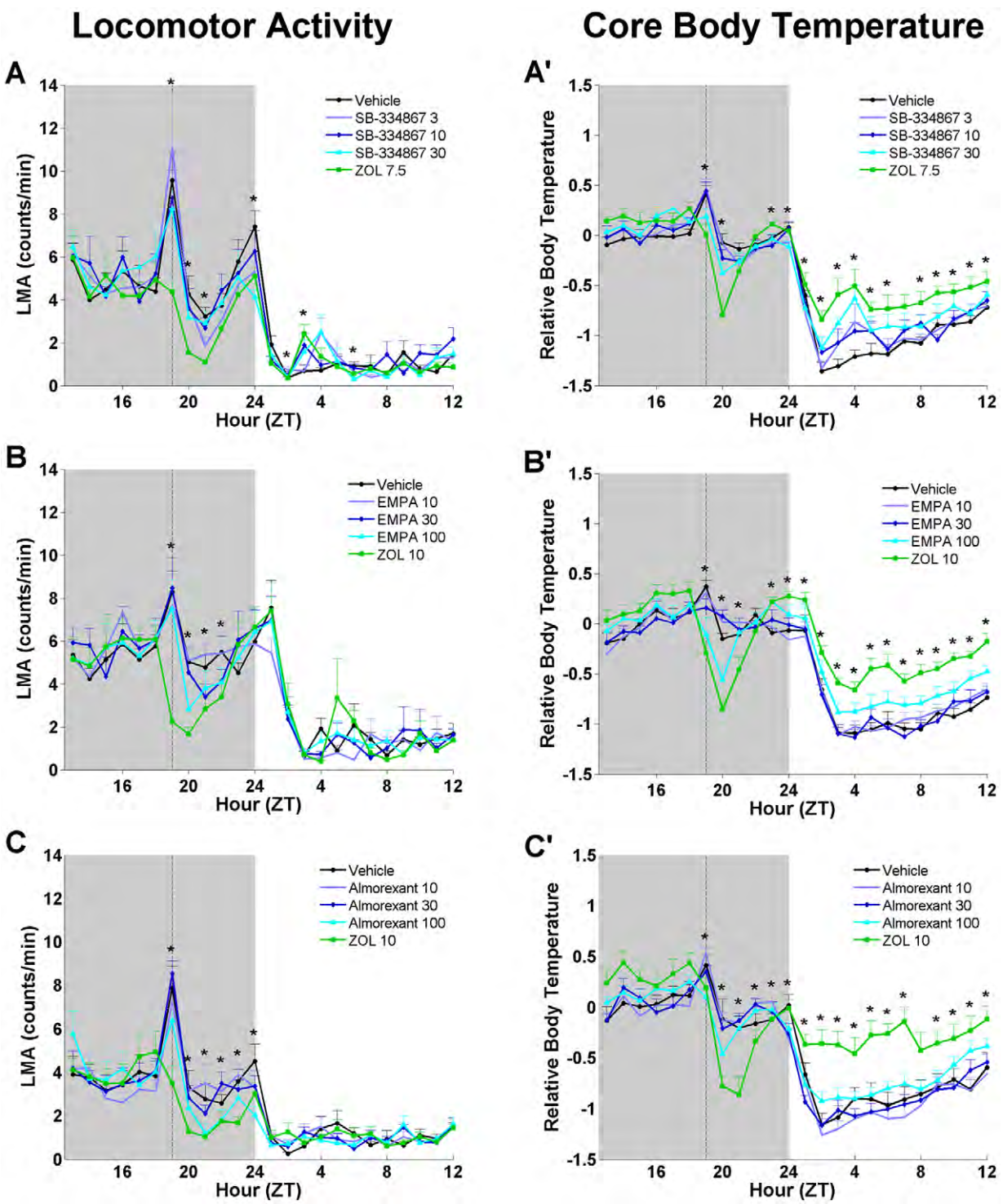

Figure 6. Average hourly LMA and relative T_{core}. LMA and relative T_{core} for 6 h prior to and 18 h after administration of SB-334867 (A), EMPA (B), and almorexant (C) as compared to zolpidem (ZOL) and vehicle. Shaded area represents the dark phase; vertical dotted line in each panel indicates the time of injection. (A) Average hourly LMA following SB-334867. (A′) The average hourly T_{core} following SB-334867. (B) The average hourly LMA following EMPA. (B′) The average hourly T_{core} following EMPA. (C) The average hourly LMA following almorexant. (C′) The average hourly T_{core} following almorexant. Data represent the mean±SEM (n = 8 rats per group). *, $p<0.05$. For detailed statistical results see Text S1.
doi:10.1371/journal.pone.0039131.g006

REM and NR sleep without rebound wakefulness and a mild change in core temperature are desirable properties of substances that induce "physiological" sleep.

On the other hand, the mechanism by which these HCRTR antagonists increased sleep duration suggests disruption of normal sleep/wake architecture. SB-334867 increased NR through a

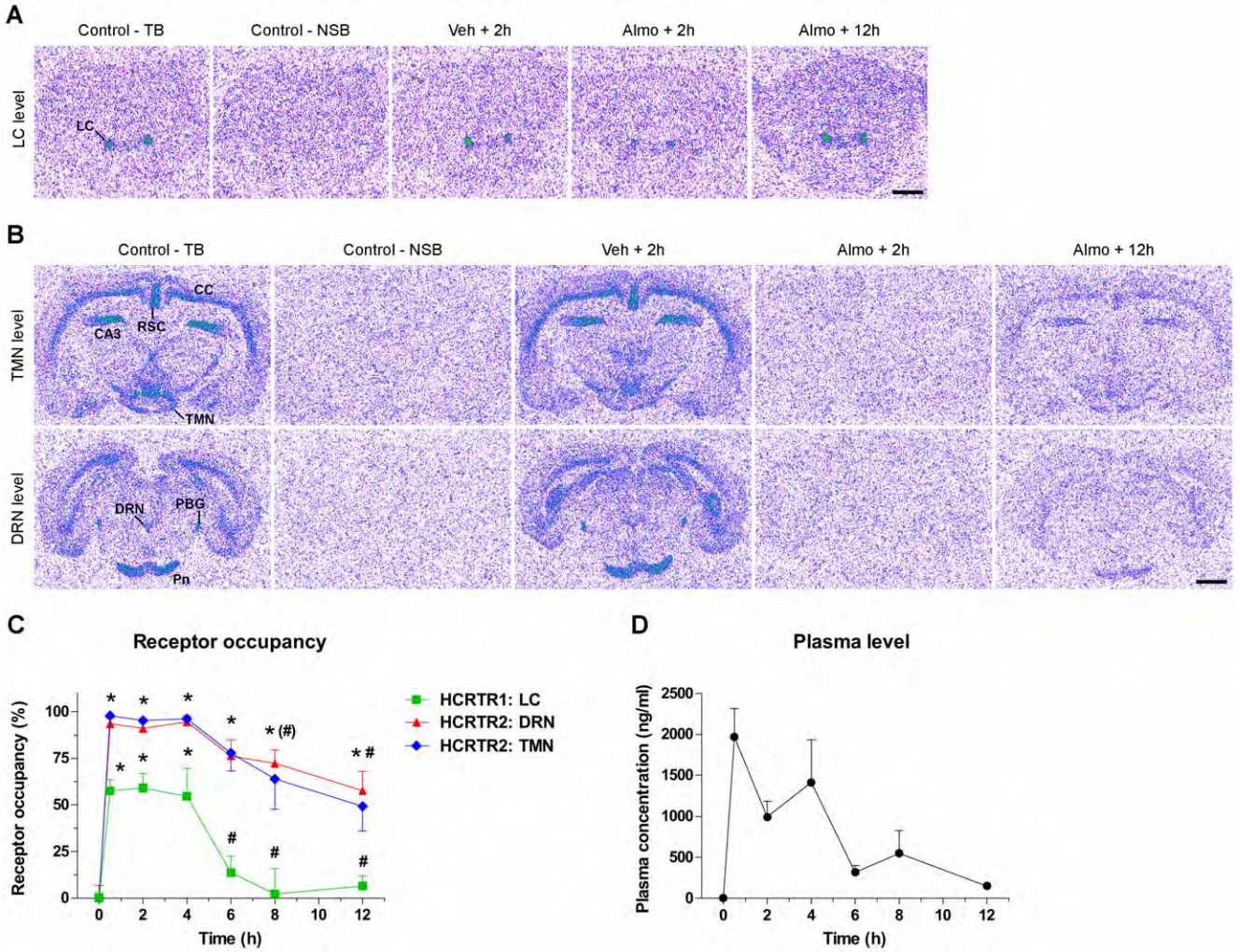

Figure 7. Time-course of HCRT1R and HCRT2R occupancies by almorexant. (A,B) Representative autoradiograms showing [3H]SB-674042 (5 nM) binding to HCRTR1 **(A)** and [3H]EMPA (1 nM) binding to HCRTR2 **(B)** in rat coronal brain sections. For both receptors, total binding (*TB*) was maximal in control animals (not injected) sampled at time 0 (*t0*). For HCRTR1 **(A)**, a clear signal was evident in the locus coeruleus (*LC*), which could be displaced by co-incubation with an excess of cold SB-674042 (10 µM) (non-specific binding, *NSB*). In contrast to vehicle administration (*Veh, 2 h*), almorexant (30 mg/kg injected intraperitoneally at ZT18) attenuated such specific signal after 2 h (*Almo, 2 h*), but not after 12 h (*Almo, 12 h*). For HCRTR2 **(B)**, signal was observed in various brain regions, including the tuberomammillary nuclei (*TMN*), cerebral cortex (*CC*), field CA3 of the hippocampus (*CA3*), retrosplenial cortex (*RSC*), dorsal raphe nuclei (*DRN*), pontine nuclei (*Pn*) and parabigeminal nuclei (*PBG*). [3H]EMPA could be displaced by co-incubation with an excess of Cp5 (10 µM) (*NSB*). HCRTR2 binding became minimal 2 h after almorexant (*Almo, 2 h*), but not after Vehicle (*Veh+2 h*), administration. After 12 h (*Almo, 12 h*), HCRTR2 binding was intermediate. Scale bars, 2 mm. **(C)** Time course of HCRTR1 and HCRTR2 occupancies by almorexant. Receptor occupancy was calculated by measuring the specific binding at various time points in the *LC* for HCRTR1, and in the *TMN* and *DRN* for HCRTR2. *, $p<0.001$ versus time 0; (#), $p<0.05$ (TMN only), #, $p<0.05$ (TMN) or $p<0.01$ (DRN), vs. time 30 min (one-way ANOVA followed by Dunnett's analysis). **(D)** Almorexant plasma concentrations. Data represent the mean±SEM (n = 5 rats per group).
doi:10.1371/journal.pone.0039131.g007

combination of small increases in both the number and duration of NR bouts that, although not significant for any particular hour, cumulatively summated into an overall significant NR increase at 4 and 6 h. For EMPA, a greater number of NR bouts underlie the overall increase in NR at the highest dose. For almorexant, NR augmentation resulted from an increased number of NR bouts without a change in bout duration, confirming previous results [32]. The increase in NR, however, was also associated with greater numbers of both W and REM bouts, particularly at the highest dose examined. Thus, although almorexant produces an overall increase in NR sleep that is greater than the other HCRTR antagonists, this is achieved through a fragmented sleep architecture. In this regard, almorexant-treated rats appear somewhat similar to *orexin* null mutant [4] or *orexin/ataxin-3* [12] mice which have disrupted sleep architecture (although these strains also exhibit cataplexy). However, the fragmentation of sleep architecture induced by dual HCRTR antagonists is consistent with the concept that the HCRT system stabilizes arousal states and minimizes the number of transitions between states [58]. Since drugs were administered to healthy animals during their active period, a more fragmented sleep architecture would be predicted. Rather than driving sleep *per se*, HCRTR antagonism seems to create a permissive neural environment for sleep to occur. Since the drive for sleep was low at the time of administration, more frequent sleep bouts without increases in bout durations could be expected.

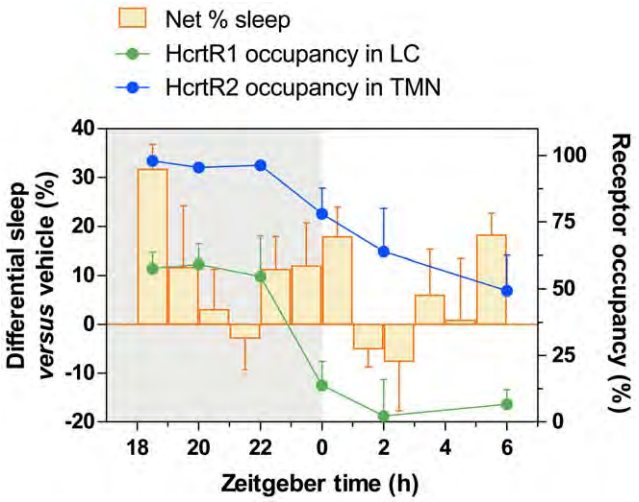

Figure 8. Net effect of almorexant on the percentage of sleep compared to HCRTR1 and HCRTR2 occupancies. The percentage of total sleep (%NR + %REM) in the vehicle-injected animals was subtracted from that of almorexant-treated rats (30 mg/kg) and was plotted over time. HCRTR1 occupancy in the locus coeruleus (*LC*) and HCRTR2 occupancy in the tuberomammillary nuclei (*TMN*) are shown in parallel. Injection occurred at ZT18. *Gray area,* dark phase; *White area,* light phase.
doi:10.1371/journal.pone.0039131.g008

Absence of Cataplexy but Facilitation of REM Sleep

One concern regarding the development of HCRTR antagonists is the possibility of inducing cataplexy as occurs in *HcrtR2* mutant dogs [3] or *HcrtR2* null mutant mice [59]. In the present study, we saw no evidence of cataplexy produced by any of the three compounds, even at the highest dose tested. However, almorexant significantly increased REM bout duration during the first hour after treatment and the highest dose – which presumably resulted in the most complete HCRTR blockade – produced 2 to 3 fold as many REM bouts during the latter half of the dark period when compared to vehicle. These observations indicate that HCRTR antagonism facilitates REM sleep occurrence, as noted by others [59].

Relationship between HCRTR Occupancy and Sleep

Whereas 30 mg/kg ip almorexant resulted in approximately 50% HCRTR1 occupancy, HCRTR2 occupancy was nearly complete in brain regions important for sleep/wake control. Moreover, while HCRTR1 occupancy declined after 4 h, HCRTR2 occupancy remained high even 12 h after treatment. While our results for HCRTR2 are consistent with a previous report, those for HCRTR1 differ [32]. A primary difference between these studies is the brain location used for determination of HCRTR1 occupancy: whereas Dugovic *et al.* used the *tenia tecta*, we measured HCRTR1 occupancy in the *LC*, an area implicated in sleep/wake control.

Figure 8 correlates RO with the net amount of sleep induced by almorexant at 30 mg/kg compared to vehicle. Since HCRTR2 occupancy is virtually 100% following this dose of almorexant while HCRTR1 occupancy is ~50%, it is likely that the stronger sleep-promoting effects observed at 100 mg/kg are due to greater HCRTR1 blockade. Figure 8 demonstrates that the sleep-promoting effects of almorexant do not simply mirror the RO data. The greatest amount of sleep occurred in the first hour after almorexant administration when occupancy of HCRTRs was maximal. Surprisingly, despite elevated occupancy of HCRTRs in subsequent hours, the hypnotic effect dissipated, suggesting that other arousal-promoting systems can overcome HCRTR blockade and produce wakefulness. In contrast, near the end of the dark phase when sleep pressure is elevated, partial HCRTR blockade was sufficient to produce sleep. These data highlight the contrasting sleep-promoting mechanisms between HCRTR antagonists and other hypnotic medications such as zolpidem. Whereas the latter compounds trigger long-lasting sleep and affect sleep intensity (sleep-inducing effect), HCRTR antagonists seem to merely antagonize wakefulness, generating conditions that allow sleep to occur (sleep permissive action).

Conclusion

Our results support the hypothesis that dual HCRTR1/R2 blockade is more effective in promoting sleep than selective blockade of either HCRTR alone. A similar conclusion was reached in a recent study of HCRT receptor knockout mice [45]. Although both HCRTR1 (SB-334867) and HCRTR2 (EMPA) antagonists produced somnogenic effects, neither promoted sleep to the levels of the dual HCRTR antagonist almorexant. Furthermore, since the lowest doses of almorexant that were sleep-promoting (30 mg/kg) bind virtually 100% of the HCRTR2s while only 50% of the HCRTR1s are occupied at that dose, the stronger sleep-promoting effects of higher doses are likely due to additional blockade of HCRTR1. These data support the notion that HCRTR antagonists are a promising avenue for sleep/wake therapeutics, with the qualifications stated above. However, given the involvement of the HCRT system in many physiological functions [9,60] including respiratory control [61,62,63,64], careful screening for side effects of HCRTR antagonists will be needed.

Supporting Information

Figure S1 Chemical structures of the compounds used in this study. Receptor selectivity is indicated into parentheses. All compounds except zolpidem are selective HCRTR antagonists. Zolpidem is a gama-aminobutyric acid (GABA) A-receptor agonist.
(TIF)

Figure S2 Hourly delta power normalized to the 24 h average vehicle control. A: 3 concentrations of SB-334867 vs. ZOL and vehicle. ANOVA is significant for treatment by time only ($F = 3.80$, $p<0.0001$). For treatment by time: **ZT19:** SB-334867 at 3 mg/kg > vehicle; ZOL >334867 at 3 and 10 mg/kg and vehicle. **ZT24:** 334867 at 3 and 10 mg/kg > ZOL; Vehicle >334867 at 10 and 30 mg/kg and ZOL **B:** 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA is significant for treatment (see legend, $F = 13.47$, $p<0.0001$) and for treatment by time ($F = 11.86$, $p<0.0001$). For treatment by time: **ZT19:** ZOL > all other conditions. **ZT20:** ZOL > all other conditions. **ZT21:** EMPA at 30 mg/kg > vehicle; ZOL > EMPA at 100 mg/kg and vehicle. **ZT22:** EMPA at 30 mg/kg > vehicle. **ZT23:** EMPA at 10 mg/kg > ZOL. **C:** 3 concentrations of almorexant vs. ZOL and vehicle. ANOVA is significant for treatment by time only ($F = 2.63$, $p = 0.0005$). For treatment by time: **ZT20:** Vehicle > almorexant at 100 mg/kg. **ZT23:** Almorexant at 10 mg/kg > vehicle. **ZT24:** Vehicle > almorexant at 100 mg/kg.
(TIF)

Figure S3 Hourly distribution of Wake Bout Duration and the Number of Wake Bouts. Wake Bout Duration (left) and Number of Wake Bouts (right) for 6 h prior to and 18 h after administration of SB-334867 (**A**), EMPA (**B**), and almorexant (**C**)

as compared to zolpidem (ZOL). Shaded area represents the dark phase; vertical dotted line shows the first h following injection. **A:** The Wake Bout Duration for 3 concentrations of SB 334867 vs. ZOL and vehicle. No significant differences were found. **A':** The Wake Bout Number for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT1-ZT6 is significant for treatment by time ($F = 1.82$, $p = 0.02341$). For treatment by time: **ZT2:** SB 334867 at 10 mg/kg and vehicle < ZOL vehicle < SB 334867 at 30 mg/kg **ZT4:** SB 334867 at 30 mg/kg and ZOL < vehicle **B:** The Wake Bout Duration for 3 concentrations of EMPA vs. ZOL and vehicle. No ANOVA's were significant. **B':** The Wake Bout Number for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 3.65$, $p = 0.01350$). ANOVA for ZT7-ZT12 is significant for treatment ($F = 4.24$, $p = 0.00647$) For treatment by time: **ZT19:** vehicle < ZOL **ZT20:** vehicle < EMPA at 30 mg/kg **ZT22:** vehicle < ZOL **ZT24:** vehicle < EMPA at 10, 30 and 100 mg/kg **ZT7:** EMPA at 10 mg/kg < ZOL **ZT11:** vehicle < ZOL **C:** The Wake Bout Duration for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 4.01$, $p = 0.01077$) and for treatment by time ($F = 2.32$, $p = 0.00234$). For treatment by time: **ZT20:** Almorexant at 100 mg/kg < ZOL **ZT21:** Almorexant at 30 and 100 mg/kg < ZOL **ZT22:** Almorexant at 100 mg/kg < ZOL and vehicle **C':** The Wake Bout Number for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 8.82$, $p = 0.00001$) and for treatment by time ($F = 2.07$, $p = 0.00769$). ANOVA for ZT7-ZT12 is significant for treatment ($F = 3.39$, $p = 0.02208$). For treatment by time: **ZT19:** vehicle < Almorexant at 30 and 100 mg/kg **ZT20:** ZOL < Almorexant at 10, 30 and 100 mg/kg **ZT21:** ZOL < Almorexant at 30 and 100 mg/kg **ZT22:**ZOL and vehicle < Almorexant at 100 mg/kg **ZT23:** vehicle < Almorexant at 100 mg/kg **ZT24:** vehicle < Almorexant at 100 mg/kg **ZT9:** Almorexant at 10 and 30 mg.kg < vehicle.
(TIF)

Figure S4 Hourly distribution of NR Bout Duration and Number of NR Bouts. NR Bout Duration (left) and Number of NR Bouts (right) for 6 h prior to and 18 h after administration of SB-334867 (**A**), EMPA (**B**), and almorexant (**C**) as compared to zolpidem (ZOL). Shaded area represents the dark phase; vertical dotted line shows the first h following injection. **A:** The NR Bout Duration for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 12.46$, $p<0.00001$) and for treatment by time ($F = 4.57$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.70$, $p = 0.00498$) and for treatment by time ($F = 3.16$, $p = 0.00004$). For treatment by time: **ZT19:** SB 334867 at 3 mg/kg and vehicle < ZOL **ZT20:** all other conditions < ZOL **ZT21:** vehicle < SB 334867 at 30 mg/kg and ZOL **ZT24:** vehicle < SB 334867 at 3 mg/kg **ZT1:** ZOL < SB 334867 at 3 and 10 mg/kg and vehicle SB 334867 at 3 mg/kg < vehicle **ZT3:** SB 334867 at 30 mg/kg and ZOL < vehicle **A':** The NR Bout Number for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT1-ZT6 is significant for treatment by time ($F = 1.81$, $p = 0.02532$). For treatment by time: **ZT1:** vehicle < SB 334867 at 3 and 30 mg/kg and ZOL **ZT4:** SB 334867 at 3 mg/kg < vehicle **B:** The NR Bout Duration for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 13.46$, $p<0.00001$) and for treatment by time ($F = 5.34$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 7.99$, $p = 0.00010$). ANOVA for ZT7-ZT12 is significant for treatment ($F = 3.03$, $p = 0.02981$). For treatment by time: **ZT19:** all other conditions < ZOL **ZT20:** all other conditions < ZOL **ZT23:** ZOL < EMPA at 10 mg/kg **ZT24:** ZOL < EMPA at 30 mg/kg **ZT2:** ZOL < EMPA at 30 mg/kg **ZT3:** ZOL < EMPA at 10 and 100 mg/kg and vehicle **ZT5:** ZOL < EMPA at 10 and 100 mg/kg and vehicle EMPA at 30 and 100 mg/kg < vehicle **ZT6:** ZOL < EMPA at 10 mg/kg and vehicle EMPA at 100 mg/kg < vehicle **B':** The NR Bout Number for 3 concentrations of EMPA vs. ZOL and vehicle. No ANOVA's were significant. **C:** The NR Bout Duration for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 16.44$, $p<0.00001$) and for treatment by time ($F = 5.34$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.83$, $p = 0.00433$) and for treatment by time ($F = 2.24$, $p = 0.00341$). For treatment by time: **ZT19:** all other conditions < ZOL vehicle < Almorexant at 100 mg/kg **ZT20:** all other conditions < ZOL **ZT21:** all other conditions < ZOL **ZT22:** Almorexant at 10 and 30 mg/kg < ZOL **ZT2:** ZOL < Almorexant at 10 and 30 mg/kg and vehicle Almorexant at 100 mg/kg < vehicle **ZT3:** ZOL < Almorexant at 10 mg/kg **ZT4:** ZOL < Almorexant at 10 mg/kg **ZT5:** ZOL < Almorexant at 10 mg/kg **ZT6:** ZOL < Almorexant at 30 mg/kg **C':** The NR Bout Number for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 12.58$, $p<0.00001$) and for treatment by time ($F = 2.41$, $p = 0.00149$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.18$, $p = 0.00890$). For treatment by time: **ZT19:** vehicle < Almorexant at 30 and 100 mg/kg **ZT20:** ZOL < Almorexant at 10, 30 and 100 mg/kg vehicle < Almorexant at 100 mg/kg **ZT21:** ZOL < Almorexant at 10, 30 and 100 mg/kg **ZT22:** ZOL and vehicle < Almorexant at 100 mg/kg **ZT23:** vehicle < Almorexant at 100 mg.kg **ZT24:** vehicle < Almorexant at 100 mg.kg **ZT1:** vehicle < ZOL.
(TIF)

Figure S5 Hourly distribution of REM Sleep Bout Duration and the Number of REM Sleep Bouts. REM Sleep Bout Duration (left) and the Number of REM Sleep Bouts (right) for 6 h prior to and 18 h after administration of SB-334867 (**A**), EMPA (**B**), and almorexant (**C**) as compared to zolpidem (ZOL). Shaded area represents the dark phase; vertical dotted line shows the first h following injection. **A:** The REM Bout Duration for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 4.40$, $p = 0.00692$) and treatment by time ($F = 2.16$, $p = 0.00500$). For treatment by time: **ZT19:** ZOL < SB 334867 at 3 mg/kg **ZT20:** ZOL < all other conditions **ZT23:** vehicle < all other conditions **ZT24:** SB 334867 at 10 mg/kg < ZOL vehicle < SB 334867 at 3 mg/kg **A':** The REM Bout Number for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment by time only ($F = 4.49$, $p = 0.00625$). For treatment by time: **ZT20:** ZOL < all other conditions **ZT24:** vehicle < SB 334867 at 30 mg/kg **B:** The REM Bout Duration for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment by time ($F = 1.71$, $p = 0.03515$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.88$, $p = 0.00015$) and for treatment by time ($F = 2.81$, $p = 0.00015$). For treatment by time: **ZT21:** ZOL < EMPA at 100 mg/kg **ZT24:** EMPA at 100 mg/kg < vehicle **ZT1:** EMPA at 100 mg/kg < ZOL all other conditions < vehicle **ZT4:** EMPA at 10 and 30 mg/kg < vehicle **ZT5:** ZOL < EMPA at 10 mg/kg **B':** The REM Bout Number for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 3.99$, $p = 0.00888$) and for treatment by time ($F = 1.96$, $p = 0.01112$). For treatment by time: **ZT20:** ZOL < all other conditions **ZT22:** vehicle < ZOL **ZT23:** ZOL < vehicle **C:** The REM Bout Duration for 3 concentrations of Almorexant vs.

ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment by time ($F = 6.91$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.45$, $p = 0.00657$). For treatment by time: **ZT19:** ZOL and vehicle < Almorexant at 10, 30 and 100 mg/kg **ZT20:** all other conditions < ZOL **ZT24:**ZOL < Almorexant at 10 and 100 mg/kg and vehicle Almorexant at 30 mg/kg < vehicle **C':** The REM Bout Number for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 9.29$, $p = 0.00007$) and for treatment by time ($F = 2.96$, $p = 0.00010$). For treatment by time: **ZT19:** ZOL and vehicle < Almorexant at 30 mg/kg **ZT20:** ZOL < Almorexant at 10 and 30 mg/kg and vehicle **ZT21:** ZOL < all other conditions **ZT22:** ZOL and vehicle < Almorexant at 100 mg/kg **ZT23:** vehicle < Almorexant at 100 mg/kg **ZT24:** ZOL and vehicle < Almorexant at 100 mg/kg.
(TIF)

Figure S6 Brain concentration of almorexant. Time course of almorexant concentration in the brain of rats injected intraperitoneally with 30 mg/kg at the mid-dark phase (same animals as in Figures 7). Data are the mean±SEM (n = 5 rats per group).
(PDF)

Figure S7 HCRTR2 occupancy in the cerebral cortex, retrosplenial cortex, pontine nuclei, and hippocampus. Data are the mean±SEM (n = 5 rats per group). *, $p<0.001$ vs. time 0; ##, $p<0.01$, #, $p<0.05$ vs. time 30 min (one-way ANOVA followed by Dunnett's analysis). Almorexant plasma concentrations (data from Figure 7) are shown for comparison.
(TIF)

Materials and Methods S1 Expanded materials and methods for both *in vitro* and *in vivo* experiments as referenced in the text.
(DOCX)

Text S1 Expanded legends for Figures 5 and 6 that include detailed statistical results.
(DOCX)

Table S1 Pharmacokinetic assessment of almorexant, SB-334867 and SB-408124 after i.v. and p.o. administration to Wistar rat.
(DOCX)

Table S2 CEREP selectivity screen in the broad radioligand binding assays were undertaken to determine the pharmacological activity of SB-334867.
(DOCX)

Table S3 Measures of state consolidation for 6 h following the administration of SB-334867.
(DOCX)

Table S4 Measures of state consolidation for 6 h following the administration of EMPA.
(DOCX)

Table S5 Measures of state consolidation for 6 h following the administration of almorexant.
(DOCX)

Acknowledgments

We thank Emmanuel Pinard, Philipp Huguenin, Thomas Hartung and Rodolfo Gasser for the synthesis, radiolabeling, and pharmacokinetic determination of the antagonists, Christophe Flament, Patrick Mortas, Karine Jeanneau, Patricia Glaentzlin, Hugues Isel, Claudia Kratzeisen and Anne Marcuz of F. Hoffmann-La Roche Ltd and William Sinko, Kristy Silveira and Alan Wilk of SRI International for excellent technical assistance and RA Sanchez of F. Hoffmann-La Roche Ltd for valuable input on the manuscript. The content of the information does not necessarily reflect the position or the policy of the U.S. Government, and no official endorsement should be inferred.

Author Contributions

Conceived and designed the experiments: SRM FGR JLM JGW TSK EB PM. Performed the experiments: SRM FGR DV EB. Analyzed the data: SRM FGR DV JLM TSK EB PM. Wrote the paper: SRM FGR TSK.

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